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Electrogenetherapy of Established B16 Murine Melanoma by

Using an Expression Plasmid for HIV-1 Viral Protein R

by

Andrea Nicole McCray

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Molecular Medicine
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long-term survival, Vpr, anti-cancer agent

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Dedication

This dissertation is lovingly dedicated to my God and my family. I have dedicated my life and research to my Lord and Savior, Jesus Christ, who has given me the wisdom and endurance to complete this project (Romans 10: 9-10). I also dedicate this work to my family: Calvin and Evelyn McCray III, Adrian McCray, and Alex McCray. Each family member has played a tremendous role in my success throughout my entire educational life. Thank you for rejoicing with me during the good times and praying for me during the more challenging times. Without the love, support, and encouragement of my family I would not have overcome the challenges associated with graduate school.

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Note to Reader

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The original dissertation is on file with the USF library in Tampa, Florida.

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Electrogenotherapy of Established B16 Murine Melanoma by Using an Expression Plasmid for HIV-1 Viral Protein R

Andrea Nicole McCray

Abstract

Novel therapies and delivery methods directed against malignancies such as melanoma, and particularly metastatic melanoma, are needed. The HIV-1 accessory protein Vpr (viral protein R) has previously been demonstrated to induce G2 cell cycle arrest as well as *in vitro* growth inhibition/killing of numerous tumor cell lines. *In vivo* electroporation has been utilized as an effective delivery method for pharmacologic agents as well as DNA plasmids that express “therapeutic” proteins and has been targeted to various tissues including malignant tumors. In this study, we assessed the ability of electroporation-mediated delivery of Vpr plasmid (pVpr) to induce growth attenuation or complete tumor regression in C57BL/6 mice with subcutaneous B16.F10 melanoma lesions. To assess the administration of intratumoral delivery of pVpr with *in vivo* electroporation, a range of Vpr plasmid dosages, electroporation parameters, and treatment days were evaluated in a subcutaneous B16 murine melanoma model. pVpr was injected directly into the tumors. Immediately following the injection, the subcutaneous tumors were electroporated. Treatment with 25 µg or 100 µg of pVpr plus electroporation on days 0 and 4 resulted in complete tumor regressions with long-term survival in 14.3% and

7.1% of the mice, respectively. In order to optimize the treatment regimen, B16 tumors were treated on days 0, 2, and 4 with 100 µg pVpr plus electroporation which resulted in 50% of the mice with complete tumor regressions and long-term survival. Additional investigations revealed intratumoral Vpr expression and demonstrated that apoptosis was the mechanism by which Vpr caused tumor regression *in vivo*. This study confirmed that treatment with 100 µg of pVpr plus electroporation led to durable complete regressions in established murine melanoma lesions. The pVpr plus electroporation treatment regimen has induced complete regressions in mice as well as resistance to tumor challenge in some of the animals. This is the first comprehensive study demonstrating the ability of Vpr, when delivered as a DNA expression plasmid with *in vivo* electroporation, to induce complete tumor regressions coupled with long-term survival of mice in a highly aggressive and metastatic solid tumor model.

Introduction

Cutaneous Melanoma

Melanoma accounts for only 4% of skin cancers but is responsible for nearly 80% of the mortality from these cancers, due to metastatic spread (1). Human melanoma develops from malignant melanocytes that travel to the epidermis (2). Differentiated melanocytes are located in the basal layer of the epithelium where they produce melanin (3). A majority of melanomas that are diagnosed are categorized as either melanoma in situ or superficial spreading melanoma because the lesion originates as a radial growth phase (2-4). However, some melanomas undergo a vertical growth phase. Nodular melanoma is the second most common form of melanoma and demonstrates vertical growth (3-5). A primary melanoma which demonstrates both a tumor thickness greater than 2 mm and vertical growth has a greater metastatic potential if left untreated than an in situ melanoma growing in radial phase. Features of metastatic melanoma include melanin in some portion of the tumor, a nest of malignant cells clumped in one region, epithelioid and spindle cells (3).

In 2002, the American Joint Committee on Cancer (AJCC) revised the staging system of melanoma, primary tumor, node, metastasis classification (6,7). According to the AJCC, stages I and II pertain to primary tumors in which disease has not spread to the lymph node. Stage III pertains to local lymph node participation and “in transit” metastases (8). Stage IV involves systemic metastases.

Many prognostic factors for cutaneous melanoma have been examined throughout the years. The most critical prognostic factors include primary tumor thickness, ulceration of tumor, number of lymph nodes involved in metastasis, sentinel lymph node detection of micrometastasis, and regions of distant metastasis (6,7). Certain prognostic factors are more crucial in predicting survival rates in comparison to other prognostic factors depending on the stage of disease.

The main prognostic factors that influence survival rates for stage I and II patients are tumor thickness and ulceration. In stage I disease, surgery can be postponed for more than three weeks after biopsy without the patient's five year outcome being adversely affected (9). Immense tumor thickness is indicative of lower survival rates (10,11). The more ulcerative a tumor appears, the lower the survival rate (12,13)

Stage III melanoma involves metastasis. Metastasis in the lymph nodes may be detected at various time points during disease: when primary melanoma is detected; after treatment of the primary melanoma; or develop without an identified primary melanoma. No matter which method/route was used for lymph node metastasis to form, survival rates are not delineated until after the metastasis is discovered (8). The greater the number of local lymph nodes with metastasis, the lower the survival rate of patients after 10 years (12,14). The Sydney Melanoma Unit/University of Alabama at Birmingham (SMU/UAB) study demonstrated that Stage III patients with one positive lymph node had 40% survival, whereas patients with greater than 5 positive lymph nodes had only 15% survival at 10 years (12). Even in Stage III disease, tumor ulceration is a serious problem in terms of percent survival rates. The important prognostic factors for stage III: number

of lymph nodes involved in metastasis, an ulcerated primary melanoma, and nodal size (6,7,12). Systemic interferon therapy enhances the survival rate of stage III patients.

Stage IV patients have more of a problem than the patients in other stages of disease. Sites of stage IV metastasis include the skin, soft tissue, or remote lymph nodes for the first sites. A secondary site of metastasis is the lungs. Brain, liver, and bone cancer can also arise from metastatic melanoma (8). In stage IV, the focus is no longer on the primary tumor as an indication for survival rates. The main prognostic factor is the anatomic location of the metastasis (6,7).

Melanoma Markers Detected in Patients

There are a variety of tumor associated markers linked to melanoma. One of these markers, tyrosinase, is an enzyme involved in the synthesis of melanin. Since tyrosinase is expressed on normal and malignant melanocytes, it is categorized as an expressed, nonmutated differentiation antigen similar to MART-1 and gp100. MART-1 is defined as Melanoma Antigen Recognized by T cells. MAGE-3 is a Melanoma Antigen Gene which has antigens expressed on only cancer cells and the testis. MUC-18 and p97 are melanoma-associated antigens.

Tyrosinase, MAGE-3, Muc-18, and p97 were found in individuals with melanoma by using a multiple-marker assay. The multiple-marker assay involves using RT-PCR to test peripheral-blood lymphocytes (PBL) for the simultaneous presence of tyrosinase, MAGE-3, MUC-18, and p97. The multiple-marker assay was first optimized in vitro by using melanoma cell lines and PBL derived from normal volunteers to examine the presence of tyrosinase, MAGE-3, MUC-18, and p97. The results demonstrated that all four melanoma markers were present in at least 80% of the melanoma cell lines and only

2 of the normal volunteers had MUC-18(15). By using the same multiple-marker assay system on PBLs taken from melanoma patients, it was demonstrated that tyrosinase, MAGE-3, MUC-18, and p97 markers were detected in the melanoma patients. As the clinical stage of disease evolved from stage I to stage IV, there was a significant correlation between the number of the aforementioned tumor markers and the stage of disease. Therefore, stages II, III, and IV melanomas had the most tumor markers. Also, proteins such as MART-1/Melan-A and gp100 have been detected in lesions from metastatic patients (16).

Diagnosis of Melanoma

Biopsy and Dermoscopy

Biopsy is a technique used to obtain samples for pathologic diagnosis. Biopsy is the main method by which suspicious lesions may be diagnosed as melanoma or nonmelanoma (17). One type of biopsy is an incisional biopsy. An incisional biopsy is performed by using a scalpel or biopsy punch to extract only a section of the tumor. During an incisional biopsy, there is removal of the most raised section of the tumor, or the darkest region if the tumor is flat (17).

A noninvasive procedure used to identify possible melanomas is dermoscopy. Dermoscopy involves transillumination of the suspected region. Immersion oil is placed onto the pigmented region and inspected by a dermoscope, which is a lens that has a transilluminating light source. This procedure enables the physician to visualize structures underneath the skin surface.

Issues Concerning the Eradication of a Solid Tumor and Prevention of Tumor Recurrence

Cancer treatment modalities such as immunotherapy must contend with the issue of cancer cells evading the immune system, thereby avoiding destruction. In one scenario, the immune system may not mount an immune response to tumor cells, even though antigen presenting cells (APCs) may have the capacity to present tumor antigens but be incapable of reaching T cell regions of the lymph nodes and fail to activate T cells (18). Another mechanism of tumor evasion is the downregulation of certain molecules such as MHC molecules or principal tumor antigens. Some tumor cells have developed a resistance to apoptosis by overexpression of anti-apoptotic proteins or downregulation of pro-apoptotic proteins (18).

The process of a melanocyte converting into melanoma may be accompanied by the loss of HLA class I and HLA class II antigens (8). Twenty-one percent of primary human melanoma cells are deficient in HLA-A2 and/or A28. However, forty-four percent of human metastatic melanoma cells are deficient in HLA-A2 and/or A28 (19).

There are various categories of human melanoma antigens that are recognized by T cells. The main divisions of melanoma antigens include antigens mainly expressed by tumor cells; melanocyte differentiation antigens; antigens that are largely expressed on tumors; and melanoma specific antigens (20). With the exception of expression on the testis, there are antigens that are mainly expressed by numerous tumors including melanoma, which are presented to T cells in the MHC Class I and MHC Class II format (20). An example of an antigen that is associated with melanoma and other tumors is MAGE. The MHC class I and class II restricted melanocyte differentiation antigens are

expressed on normal and neoplastic melanocytes. Melan-A/MART1 is the more dominant immunogen among all the melanocyte differentiation antigens. The antigens that are largely expressed on tumor cells are recognized only by MHC class I restricted T cells and can be present on both normal and cancerous tissues. p15 and p53 are good examples of antigens that are largely expressed on tumor cells (20).

The melanoma specific antigens are presented in a MHC class I format to T cells. An important melanoma specific antigen is tyrosinase-related protein-2 (TRP2). If one of the common melanoma antigens were very immunogenic, it would possibly be effective in a melanoma vaccine (20). However, even though melanoma patients may have a cytotoxic T cell response following previous exposure to melanoma antigenic peptides in the form of a vaccine, there was no link with clinical responses (21-23). Low survival rates have been associated with a lack of lymphoid infiltration (24,25).

Many cancer cells possess self antigens that the immune system will not recognize and attack. Even if cancer cells have some tumor antigens with dominant epitopes, it remains difficult to find the right combination of antigens such as melanoma antigens to produce an effective melanoma vaccine. Even though tumor associated antigens (TAAs) can elicit an immune response, one needs the right combination of TAAs for efficacy. MART1 and gp100 are two important TAAs for human melanoma.

The major problem with controlling the growth of many solid tumors including B16.F10 melanoma is that they are poorly immunogenic. Some melanoma tumors express CD95L also known as FasL (26-28). Some tumor cells elude the immune system by using their CD95L to attach to CD95 receptor on immune cells and induce cell death (apoptosis) of the immune cells (29). CD95 and CD95L already exist on B16 melanoma

cells. However, exposure to IFN- γ significantly enhanced the expression of CD95 and CD95L on B16 cells. B16 cells which possess both CD95 and CD95L are primed for apoptosis (29). In addition, B16 cells do not express MHC class II molecules at all. However, B16 cells express extremely low quantities of MHC class I (30). Both the MHC class I and class II molecules can be expressed on B16 cells once the cells are exposed to IFN- γ (30).

The B16.F10 tumor model is an excellent tumor model to examine the intricacies of human tumor development/progression and treatment strategies since the B16.F10 tumor is poorly immunogenic. Treatment strategies would have to effectively overcome the lack of immunogenicity to eradicate the tumor and provide long-term protection against recurrence.

Alternative Adjuvant Treatment Strategies for Melanoma

Interventional treatment options for melanoma other than surgery are relatively limited and underscore the need for the development of novel efficacious therapies. Even after surgical removal of the primary melanoma, patients who had primary melanoma greater than 4 mm or any form of metastasis are at increased risk of tumor recurrence. Interferon alpha-2b is administered to patients who have a high risk of melanoma recurrence (31,32). The FDA approved the use of high dose interferon alpha for adjuvant therapy after surgery in patients with stage IIB and stage III melanoma. Thus far, high dose interferon alpha-2b treatment provides significant relapse free survival but cannot establish overall survival benefits. Since the FDA approved the use of interferon alpha-

2b, many cancer centers have utilized this therapy. However, there is no widespread consensus of using interferon alpha-2b throughout the U.S.

IL-2 is another alternative treatment for patients with metastatic melanoma (33). Even though IL-2 therapy results in rare complete responses (i.e. 8% complete responses or complete tumor regressions), it is one of the only treatments for metastatic melanoma. Both interferon alpha and IL-2 are given at doses that have a high potential for toxicity. Some treatments offer enhanced overall survival for melanoma patients but not necessarily regression of the tumors. In the animal model, especially in the B16.F10 tumor model, significant growth inhibition is customarily the only attainable goal for treatment strategies.

Instead of focusing attention on using immunotherapy to recruit the immune system to eradicate tumors, it may be more beneficial to shift attention to delivering an agent that will eradicate tumors through intracellular mechanisms and then recruit immune cells to establish a long-lasting anti-tumor response. One such agent is the Vpr protein, from HIV-1, which causes apoptosis in various cell lines including cancer cells (34,35). The other issue concerning eradication of a solid tumor is the choice of the delivery mechanism. A well-accepted non-viral delivery mechanism is electroporation. Therefore, in this study, *in vivo* electroporation was used in order to effectively deliver plasmid encoding Vpr to B16 solid tumors.

HIV-1 and Vpr

HIV-1 is the etiological agent of AIDS in humans. HIV-1 is a disease that is obtained through contact with infected blood and sexual contact with an infected person.

It is a lentivirus which incorporates its provirus into the genome of the host and uses the host's cellular machinery to produce its viral proteins (36). Unlike other retroviruses, HIV-1 also has six accessory genes in addition to its customary structural genes, *gag*, *pol*, and *env* (36,37). One accessory gene that is conserved throughout HIV-1, HIV-2, and SIV is *vpr*.

Research has shown that HIV-1 causes cell growth arrest and differentiation in cell culture. Following transfection of a HIV-1 plasmid into TE671 embryonal rhabdomyosarcoma cells, cell growth arrest occurred within days (38). After almost six days, the HIV-1 transfected cells were tremendously enlarged in size, completely viable, and did not replicate (38). The transfected TE671 cells continued to show viability *in vitro* for at least 3 weeks. Levy et al. (1993) provided the first evidence that HIV-1 has the capability of inducing cell cycle arrest without cell death as well as the capacity of inducing cell differentiation.

After various HIV-1 structural genes as well as accessory genes were tested, it was found that Vpr was responsible for cell growth arrest and differentiation *in vitro*. In fact, following exposure to an HIV-1 genome which lacked *vpr*, transfected rhabdomyosarcoma cells did not experience growth arrest or substantially differentiate (38). To examine whether other transformed cells would undergo growth arrest and differentiation, Levy et al. (1993) transfected human and canine osteosarcoma cells with *vpr* plasmid. The *vpr*-transfected osteosarcoma cells experienced cell growth arrest without differentiation.

The functions of HIV-1 Vpr are diverse. Depending on the cell type, the functions of Vpr vary: cell growth inhibition; G2 arrest; influence on tumor growth; differentiation;

virus replication in macrophages and non-dividing cells; and transcriptional activation. Some of the functions of Vpr are beneficial for the survival of HIV within the host and other functions are tailored for dysregulation of cellular processes in cells other than host cells i.e. cancer cells. Wong-Staal et al. (1987) made the first assessment that the R open reading frame encoded for a protein (39). Wong-Staal et al. (1987) translated *vpr* in bacteria and discovered that approximately forty percent of HIV infected people had antibodies against the Vpr protein, demonstrating that a Vpr protein was produced *in vivo*. During *in vitro* cell culture, virus production is undetectable in HIV-infected peripheral blood cells unless the cells are stimulated by antigen, mitogen, or cytokines (40-43). *vpr* augments HIV and SIV replication in T cells and macrophages (44-48). Ogawa et al. (1989) provided the first evidence that Vpr may be a positive attribute in terms of HIV replication (46). Data demonstrate that HIV-1 *vpr* is sufficient to cause differentiation and growth inhibition in the human rhabdomyosarcoma cells, TE671 (38). The *vpr*-transfected TE671 cells underwent differentiation as demonstrated by the cells becoming large in size, developing branched processes, and being multinucleated. It was noted that these differentiated cells did not replicate after transfection. The *vpr* gene also causes differentiation and growth inhibition in various cell lines such as rhabdomyosarcoma line RD and osteosarcoma line D17 (38).

Animal Models for HIV-1

The only animals capable of being infected with HIV-1 are humans and chimpanzees. However, there is currently no reliable animal model for a naturally occurring HIV-1 infection. Data did show that chimpanzees could be infected with relatively low doses of HIV, yet there was no long-term damage to the chimpanzees'

immune systems (37). In a rare occurrence, high viral loads and a CD4⁺ T cell depletion to 100 cell/μl range did arise in one chimpanzee almost a decade after being exposed to two different HIV strains (HIV-1_{SF2} and HIV-1_{LAV-1b}). Due to the fact that chimpanzees are an endangered species that are incapable of progressing to disease in a timely manner following exposure of virus isolates, they are not sought after as an animal model for HIV-1 immunodeficiency (49).

Researchers have developed transgenic mice that express HIV-1 genes. These HIV-1 transgenic mice have copies of proviral DNA in all cells, which characterizes the *in vivo* process of the postintegration stage of the HIV-1 life cycle (37). Transgenic mice have viral genes under the direction of cell or tissue-specific promoters. There are Vpr transgenic mice.

Functions of Vpr

Apoptosis

The C-terminal domain of Vpr has a consequence on the mitochondria as evidenced by the expulsion of cytochrome C and apoptosis-inducing factor (AIF) which induce apoptosis (50). Also, apoptosis arises from the interaction of Vpr with the adenine nucleotide translocator (ANT) which leads to membrane permeabilization of the mitochondria (50). Bcl-2 is an anti-apoptotic molecule which impedes mitochondrial pore formation by interacting with ANT to hinder the binding of Vpr with ANT (51). However, in intact cells, Vpr protein can overcome the anti-apoptotic property of Bcl-2 (52).

Vpr is a multifaceted protein that induces apoptosis via a caspase-dependent as

well as a caspase-independent mechanism. AIF is a hallmark of the caspase-independent approach to apoptosis (53). In contrast to caspase-independent apoptosis, Vpr causes apoptosis through caspase-9 activation. Vpr does not operate via the Fas/FasL pathway (52). p53 is not a requirement for Vpr-mediated apoptosis even in human tumor cells (34,54).

In rodent cells, Vpr protein induces growth inhibition via G₁ arrest and ultimately apoptosis in a short span of time which leaves cells that can slowly persist in growth (55). G₁ arrest as a consequence of Vpr exposure is a more recent concept that is seen in rodent cells which have been transfected with Vpr plasmid (55). Throughout the literature primate cells have been examined and Vpr-mediated apoptosis has either followed G₂ arrest or has been independent of Vpr induced G₂ arrest. Data suggests that Vpr causes apoptosis via caspase-9 activity in both rodent and primate cells. The selection of the cell cycle arrest phase is a species restricted phenomenon, but not the occurrence of apoptosis (55).

Localization of Vpr

Endogenous Vpr Protein

There are a few sources for HIV-1 Vpr: virion-borne Vpr, endogenous synthesis of Vpr, and extracellular Vpr. Vpr in the serum is most likely bound to either viral Gag or anti-Vpr antibodies (56-58). The Gag protein is essential for the assimilation of Vpr protein into newly developing viral particles (57-59). It is still a question of whether extracellular Vpr occurs due to disintegration of infected cells or to release from infected cells (60).

Cells that have been transfected with plasmid encoding *vpr* express Vpr protein within the cells at sufficient levels. Apoptosis has been observed in various cell lines such as tumor cell lines after exposure to Vpr protein. Previous research has focused on cells derived from primates including human cancer cell lines to attest to the apoptotic activity induced by Vpr. However, even in rodent cells, Vpr protein causes apoptosis via the degradation of mitochondria and the activation of caspase-9 (55). Rodent cells derived from hamster and mouse such as BHK and NIH3T3 cells undergo apoptosis after transfection with Vpr plasmid (55).

Extracellular Vpr Protein

There are various transformed cell lines in which Vpr protein was exported after transfection with the *vpr* gene. These transformed cell lines are T cells (H9, Sup T-1), monocytes (HL60, THP-1), muscle cells (TE671, RD), and neuroblastoma cells (SK-N-MC) (56). There are several studies in which purified intracellular Vpr protein penetrates the cells (61,62).

Bystander Effect

HIV-1 soluble factors such as Vpr have always played a role in the bystander effect in terms of killing non-Vpr expressing cells during an infection. Studies have shown various uninfected cells that succumb to apoptosis during HIV infection such as uninfected CD4⁺ T cells, thymocytes, lymph node cells, CD8⁺ T cells, B cells, macrophages, natural killer cells, and dendritic cells (63-77). The Vpr-mediated bystander effect is not only demonstrated in primate cell lines or cells involved in active infection. Vpr protein induces apoptosis in Vpr -transfected rodent cells as well as non-transfected rodent cells grown in the same culture (55).

Purified Vpr protein can penetrate cells *in vitro* (61,62). If purified Vpr can penetrate cells simply by being in the presence of the cells, there is no reason not to speculate that Vpr derived from a plasmid can be secreted from transfected melanoma cells within an established tumor to other cells within that tumor. In this manner, the enhanced delivery method of intratumoral injection of plasmid encoding Vpr plus electroporation could lead to Vpr protein being secreted throughout the tumor and leaving a trail of apoptotic cells in its path. Vpr protein can create ion channels in cell membranes of live cells (78). Apoptotic attributes occurred in whole cells and purified mitochondria after exposure to extracellular Vpr (50,51).

Expression Level Determines Apoptotic or Anti-Apoptotic Attributes of Vpr

Even though the majority of studies point to the apoptotic role of Vpr in a variety of cell types, there are a minority of studies which indicate an anti-apoptotic role for Vpr. During the natural course of HIV-1 infection, it is predicted that Vpr expression is low in order to facilitate survival of the virus. The anti-apoptotic aspect was exhibited in the early stage of HIV-1 infection (79). Evidence indicates an anti-apoptotic property of Vpr when Vpr is expressed at low levels. CD4⁺ T Jurkat cells which continually expressed low quantities of Vpr were able to resist cell death after exposure to apoptosis-inducing reagents (80). Also, HEP-2 cells which continually expressed low quantities of Vpr demonstrated an anti-apoptotic feature (81). Conversely, evidence demonstrates that high Vpr expression causes apoptosis (54,82-85).

Electroporation

In Vitro Electroporation

Electroporation is a transfection method that uses the application of electric pulses (i.e. electric field) to cause transient permeability of the cell membrane and permits drugs and plasmids to cross the cell membrane (86,87). DNA was successfully transferred *in vitro* to murine lyoma cells which marked the first gene transfer into mammalian cells using electroporation (86,87). Neumann et al. (1982) greatly enhanced the electrically-mediated uptake of DNA in the murine lyoma cells by better optimizing the electric field strength conditions (high electric fields). The optimum field strength for enhanced DNA transfer was 8 kV/cm, 5 μ s. Further development of this approach led to studies demonstrating that electric fields could be used to effectively administer a chemotherapeutic reagent to mammalian cells. The investigators used 1200 V/cm to deliver chemotherapeutic drugs to B16.F10 cells *in vitro* (88). *In vitro* electroporation enhanced the cytotoxic impact of chemotherapeutic drugs such as bleomycin and cisplatin on various cell lines especially the B16.F10 cells. Today, laboratories use *in vitro* electroporation as a standard procedure for either transfecting cells or transforming bacteria with plasmid.

In Vivo Electroporation

Electrochemotherapy

Electrochemotherapy (ECT) is the treatment of tumors via the administration of chemotherapeutic agents in conjunction with electric pulses. Bleomycin is the preferred chemotherapeutic agent that has been administered in conjunction with electroporation in

ECT preclinical studies which examined tumor growth inhibition in various solid tumor models (89-92). Previous ECT experiments have demonstrated that bleomycin has the greatest decrease in drug concentration necessary to elicit an anti-tumor response (93).

Our laboratory examined an ECT protocol that delivered bleomycin intratumorally to B16 tumors as opposed to the traditional intravenous delivery of bleomycin prior to electroporation (94). The findings of this study demonstrated that intratumoral administration of bleomycin followed by electroporation yields significantly increased survival of ECT-exposed mice, B16 tumor growth delay, and high objective response rates which were mainly due to complete responses. Also, the intratumoral delivery of bleomycin administered between 30 minutes before electroporation and 3 minutes following electroporation offered a better window of opportunity for optimal electrochemotherapy than the previous intravenous method (94). Even with the improved intratumoral ECT technique, ECT offers short-term tumor regressions in established B16 tumors with a high incidence of recurrence by day 25 post-treatment (90,94-96). One plausible explanation for tumor-free ECT-exposed mice experiencing tumor recurrence in this very aggressive tumor model is that there was residual disease that resulted from microscopic tumor cells next to or beneath the original treatment site (94). Usually after ECT treatment of subcutaneous B16 melanoma, there is no long-lasting immunity that will provide resistance to future tumor challenge (90,94,96).

Additional research combined the methodologies of intratumoral ECT with intratumoral plasmid delivery plus electroporation (95). GM-CSF plasmid plus electroporation treatment was performed 24 hours after ECT and resulted in one out of eight mice achieving a complete B16 regression and resisted challenge with B16 cells in

the opposite flank for an extra 100 days (95). Sustained B16 tumor regressions were also obtained following peritumoral treatment with electrochemotherapy combined with IL-2 plus electroporation. In addition, tumors such as mammary tumors and squamous cell carcinoma regressed as a result of treatment with IL-12 plasmid plus ECT (97). The next phase of *in vivo* electroporation was to use electroporation to enhance the delivery of plasmid expressing a wide range of proteins but mainly cytokines.

Plasmid DNA Delivery plus Electroporation

In vivo delivery of plasmids by electroporation has been used to treat tumors in animal models. IL-2 or IL-12 plasmid delivered with *in vivo* electroporation had an impact on slowing B16 tumor growth (98). IL-2 or IL-12 plasmid was administered with or without electroporation on only one treatment day. IL-2 or IL-12 plasmid plus electroporation caused a significant tumor growth delay of about five to fifteen days in comparison to IL-2 or IL-12 plasmid intratumorally delivered to the B16 tumor without electroporation. Plasmids encoding for IL-12 and IL-18 delivered with electroporation inhibited B16 tumor growth in mice (99). When the combination of IL-12 and IL-18 plasmids was administered to the B16 tumors via electroporation, mice had significant longevity as well as significant reduction in tumor volume in comparison with mice that were given IL-12 and IL-18 plasmids without electroporation.

There are only a few studies in which a cytokine expression plasmid has caused complete regression of a subcutaneous B16 tumor. Treatment with IL-12 plasmid plus electroporation caused established B16.F10 melanoma lesions to completely regress which resulted in high cure rates (100,101). The electrode design, the electric field

strength, and the treatment days were the major parameters involved in the anti-proliferative activity of the electrically-mediated delivery of IL-2, IL-12, and IL-12/IL-18 plasmids. Therefore, the studies in which over 40% of the mice had complete regression resulted from the use of a penetrating electrode that rotated 1500 V/cm around the tumor on multiple treatment days (100,101). The aforementioned parameters will have to be determined in any future electrogenetherapy protocol.

Objectives

The HIV-1 Vpr protein has a variety of roles in cell cycle arrest and apoptosis of numerous cell types especially cancer cell lines *in vitro*. However, the *in vivo* activity of HIV-1 Vpr has not been closely examined in terms of tumor growth inhibition and tumor regression in a solid tumor model particularly in the context of a non-viral delivery method. For this reason, studies were initiated to examine the effects of electrically-mediated intratumoral Vpr plasmid delivery into subcutaneous B16 melanoma in C57/BL6 mice. The B16.F10 melanoma grows very aggressively and has a deficiency in MHC I molecules which makes this tumor poorly immunogenic (29). Therefore, a successful Vpr plasmid plus electroporation treatment regimen against B16 melanoma should work at the early time points after initiation of treatment and cause long-term tumor-free survival in treated mice.

The hypothesis of this work: *Intratumoral delivery of a Vpr expression plasmid administered with in vivo electroporation will lead to growth inhibition and regression of established B16.F10 melanoma in syngeneic C57BL/6 mice.*

This is the first description of the ability of a plasmid expressing HIV-1 Vpr, when delivered by *in vivo* electroporation, to cause complete regression of an aggressive metastatic tumor such as melanoma. In order to characterize the anticancer mechanism of Vpr, the following aims have been proposed.

Specific Aim 1

To Determine the Optimal pVpr Dosage and Electroporation Parameters Required for Tumor Reduction

Electrogenetherapy enhances protein expression following delivery of a plasmid that encodes the protein of interest. In this manner, Vpr protein would be more effectively expressed once pVpr has been administered by *in vivo* electroporation. Several dosages ranging from 25 µg to 200 µg of pVpr in combination with electroporation will be examined in order to determine the appropriate dosage of pVpr that causes optimum tumor growth inhibition and/or regression. In addition to the proper pVpr dosage, the optimum electroporation parameters will be incorporated into the protocol. After appropriate treatment, tumor growth rates will be evaluated based on tumor volume calculations. In order to substantiate that Vpr protein is contributing to the tumor inhibition and/or regression, it will be necessary to examine Vpr expression following electrically mediated delivery of pVpr to tumors. Vpr protein will be detected by immunohistochemical staining of tumors excised at time points prior to regression.

Specific Aim 2

To Elucidate a Mechanism Regarding the Ability of Vpr to Inhibit Tumor Growth In Vivo

Previous *in vitro* studies have shown that Vpr induces apoptosis in a variety of cancer cells; similarly pVpr plus electroporation may reduce B16 tumor volume in mice through induction of apoptosis. The elucidation of the tumor inhibitory mechanism will involve the use of the TUNEL assay. The TUNEL assay will be used to determine

apoptosis in tumor-bearing mice after the mice have received various pVpr treatment regimens.

Specific Aim 3

To Investigate whether pVpr Induces an Immune Response, which would allow the Treated Mice to be Resistant to Tumor Challenge

Previous research has demonstrated that following electrically- mediated delivery of certain chemotherapeutic agents or cytokine expressing plasmids, the B16.F10 tumors recur at the original site of regression. However, there have been successful treatments administered with electroporation that have yielded long-term complete B16 tumor regression without tumor recurrence, cured mice. In the scenarios in which long-term tumor regression occurs, mice are challenged with parental B16.F10 cells on the flank opposite to the one which was originally treated. If cured mice are able to resist challenge with parental cells then an immune system component may be associated with the treatment. If any mice are cured following treatment with pVpr plus electroporation, they will be injected with B16.F10 melanoma cells on the opposite flank and monitored for tumor growth at the challenge site.

Methods

Mice

C57BL/6 mice, the murine strain syngeneic for the B16.F10 melanoma tumor cell line, were used in this study and were purchased from the National Cancer Institute (NCI). Mice were housed and maintained during this study in accordance with AALAM guidelines.

Tumor Cells

The B16.F10 murine melanoma cell line (CRL 6475) was originally purchased from ATCC and was maintained for studies as monolayers in culture in 90% McCoy's medium supplemented with 10% fetal bovine serum (FBS). For the preparation of single cell suspensions, monolayer of cells were detached from flasks using trypsin-EDTA (0.05% trypsin-0.53 mM EDTA), centrifuged, washed and resuspended at the proper concentration for injection in the tumor induction experiments described below.

Subcutaneous Tumor Implantation and Measurements

Tumors were induced by subcutaneous injection of 1×10^6 B16.F10 cells which were prepared as described above (viability of cells was greater than 90% as measured by Trypan blue exclusion) into the left flanks of C57BL/6 mice. Tumors were allowed to

grow to the appropriate size of at least 29 mm³ but could be larger (depending on the experiment) before commencement of the treatment regimen. A tumor volume of at least 29 mm³ has been determined to be an ideal minimal size for intratumoral injection since the administered treatment volume is retained effectively within the lesion with no significant leakage, providing confidence that the entire dose had been administered. Tumor volumes were measured before and at periodic intervals following treatment using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). Using those measurements the tumor volume was calculated by the formula: $V = ab^2 \times \pi/6$. The mice were followed in each experiment until the defined end-point was reached or until tumor volume was determined to be 1300 mm³ (at which point any mice had usually succumbed to tumor burden or were euthanized due to the size of the tumor).

Plasmid DNA

The pVpr was a gift from Dr. David Weiner. pVpr encodes the Vpr protein under the control of a CMV promoter. pcDNA3.1 is the non-coding control plasmid (Invitrogen). Plasmids were prepared using an endotoxin-free procedure (Qiagen, Valencia, CA) and suspended in sterile physiological saline.

Anesthesia

Mice were anesthetized by putting them in an induction chamber permeated with 2.5% isoflurane and 97.5% oxygen. Anesthesia was supplied to the mice via a rodent mask.

Intratumoral Delivery of Plasmid plus *In Vivo* Electroporation

Tumors were treated intratumorally with the specified dosage of the Vpr plasmid (pVpr) or control / backbone plasmid vector (pcDNA3.1) depending on the experiment. Subsequently, tumors from the appropriate groups were subjected to *in vivo* electroporation using a custom-made applicator containing 6 penetrating electrodes that was inserted into the tissue around the tumor. Depending on the treatment group, electric pulses were administered using a BTX T820 pulse generator (BTX/Harvard Apparatus, Hollister, MA) and autoswitcher (Genetronics, San Diego, CA). The optimum electroporation parameters were 1500 V/cm, 100 μ s, 6 pulses. These electroporation parameters were previously described in other studies by our group and were selected because of their less stringent parameters coupled with the ability to elicit a significant biological effect.

Treatments were administered on selected days depending on the electroporation protocol used in the experiment. For the treatment groups P+ or P- indicates with or without treatment with the pVpr and E+ or E- indicates with or without electroporation, respectively. V+ or V- indicates with or without the control “backbone” vector (pcDNA3.1), respectively.

The mean tumor volume was calculated for each group at selected time points after the treatment regimen. Additional quantitative measurements made during the study were fold increase in tumor volume compared to day 0 as well as percent of mice undergoing complete tumor regression coupled with long-term survival.

Immunohistochemistry

Tumors were excised at selected time points after treatment and snap frozen.

Tumors were sliced into 5 μ m sections. Sections were fixed in ice cold methanol for 10 minutes. Non-specific antigens were blocked in 3% BSA/PBS/Triton X-100 for one hour at room temperature. Sections were blocked in mouse BD Fc Block in 3% BSA/PBS/Triton X-100 for one hour (BD Pharmingen). The sections were stained with the polyclonal Vpr antibody (diluted 1:2000) for one hour at room temperature. Then sections were incubated with Alexa Fluor 594, a PE-conjugated secondary antibody (diluted 1: 8000) (Molecular Probes, Inc.). Sections were stained with Hoechst to identify the nuclei. The sections were visualized using a fluorescent microscope.

TUNEL Assay

For each treatment group, the same tumor sample was used to prepare consecutive sections for immunohistochemistry and the TUNEL assay. TUNEL refers to Terminal transferase dUTP nick end labeling which allows for the detection of apoptosis. Sections were fixed in ice cold methanol for 10 minutes. Apoptosis was assessed using the TACS in-situ kit TdT-Fluorescein (R & D Systems). Tumor sections were treated with proteinase K for one hour at room temperature. A TUNEL reaction mixture containing TdT biotinylated nucleotides was added to each section and incubated in a humidified chamber for one hour at 37 °C. The biotinylated nucleotides were incubated with a streptavidin-fluorescein conjugate and visualized using a fluorescent microscope. The sections were also stained with Hoechst.

Statistical Analysis

Statistical analysis was conducted using the Student's t-test. $p < 0.05$ was determined to be statistically significant.

Results

Specific Aim 1

*To Determine the Optimal pVpr Dosage and Electroporation Parameters
Required for Tumor Reduction*

Preliminary Studies on Vpr Expression and B16 Tumor Growth Inhibition

Numerous preliminary experiments were conducted in order to set parameters to ascertain pivotal information that would lay the foundation for addressing the specific aims of this research project. In many instances, the total number of animals used in the preliminary experiments was small because these experiments were merely designed to test a hypothesis such as whether there would be protein expression after electrically-mediated delivery of plasmid or whether B16 tumor growth inhibition would occur as a consequence of electrically-mediated delivery of pVpr. The experiments for the optimal pVpr regimens have higher n values ranging from 10-14 mice after duplicate experiments were conducted.

The preliminary experiments addressed a wide range of issues such as the selection of the most effective electroporation parameters for delivery of pVpr and Vpr protein expression. The initial experiments involved selecting the parameters for the appropriate pVpr dosage, effective electroporation conditions, elevated Vpr expression,

and optimal treatment days that would cause the most reduction of B16 tumor in the mouse model. In particular, B16 tumors were treated with dosages ranging from 25 µg to 200 µg of pVpr plus electroporation in order to determine which dosage would yield the most tumor growth inhibition. Four different electroporation parameters were examined in order to determine which parameters would cause the highest Vpr protein expression. The electroporation parameters were as follows: 100 V/cm, 150 ms; 200 V/cm, 20 ms; 800 V/cm, 5 ms; and 1500 V/cm, 100 µs. Similar electroporation parameters that were used to confirm Vpr expression were also used to determine which parameters would effectively deliver pVpr to the B16 tumor.

Another key factor in establishing a pVpr plus electroporation treatment protocol was the schedule of treatment days. Preliminary experiments involved using the initial treatment regimen in which treatments were administered on days 0 & 4. Following some revisions in the schedule, tumor-bearing mice were later treated daily or on alternating treatment days to obtain more tumor reduction and/or tumor regression than the day 0 & 4 treatment regimen. The individual parameters examined in the preliminary experiments to effectively deliver an optimal pVpr dosage with the appropriate electroporation conditions on particular treatment days would be combined for future experiments to electrically-mediate the delivery of pVpr and yield the highest Vpr expression along with tumor reduction and/or regression. The key for the following experiments: + or - = with or without, P= pVpr, V= pcDNA3.1 (non-coding vector), and E = electroporation.

Confirmation of Intratumoral Expression of Vpr in B16 Melanoma Lesions

Initial studies were conducted to confirm Vpr expression and to determine the kinetics of protein expression. As part of this evaluation, different electroporation

parameters (i.e. field strength and pulse width) were tested. Tumor-bearing mice were treated with a single injection of pVpr followed by electroporation. One-hundred micrograms of pVpr was chosen to study the time course for Vpr protein expression. Following treatment, four tumors per group were excised at day 2 and day 4. The tumors were subjected to immunohistochemistry using the polyclonal Vpr antibody. The treatment groups were as follows: Group 1: P-E+ saline 1500 V/cm, 100 μ s; Group 2: P+E- 100 μ g pVpr; Group 3: P+E+ 100 μ g pVpr 100 V/cm, 150 ms; Group 4: P+E+ 100 μ g pVpr 200 V/cm, 20 ms; Group 5: P+E+ 100 μ g pVpr 800 V/cm, 5 ms; Group 6: P+E+ 100 μ g pVpr 1500 V/cm, 100 μ s. Figure 1 depicts intratumoral Vpr staining of a tumor excised on day 2 post-treatment. At day 2 post-treatment, the saline + 1500 V/cm, 100 μ s treated tumors have non-specific staining (Figure 1A). There is Vpr expression after delivery of pVpr without electroporation (Figure 1B). In comparison to pVpr delivered without electroporation, there is greater distribution of Vpr protein throughout the tumor section following delivery of pVpr plus 1500 V/cm, 100 μ s (Figure 1F). The other electroporation conditions yielded minimal Vpr protein expression (Figure 1C-E). Figure 2 depicts intratumoral Vpr staining of a tumor excised on day 4 post-treatment. At day 4 post-treatment, there is only non-specific staining for the tumors treated with saline plus 1500 V/cm, 100 μ s (Figure 2A). The tumors treated with pVpr without electroporation did not have Vpr expression at day 4 post-treatment (Figure 2B). At day 4, there is still no detectable Vpr protein expression for any of the other electroporation conditions (Figure 2C-E). By day 4 post-treatment, the tumors treated with 100 μ g pVpr plus 1500 V/cm, 100 μ s no longer demonstrate Vpr protein expression (Figure 2F).

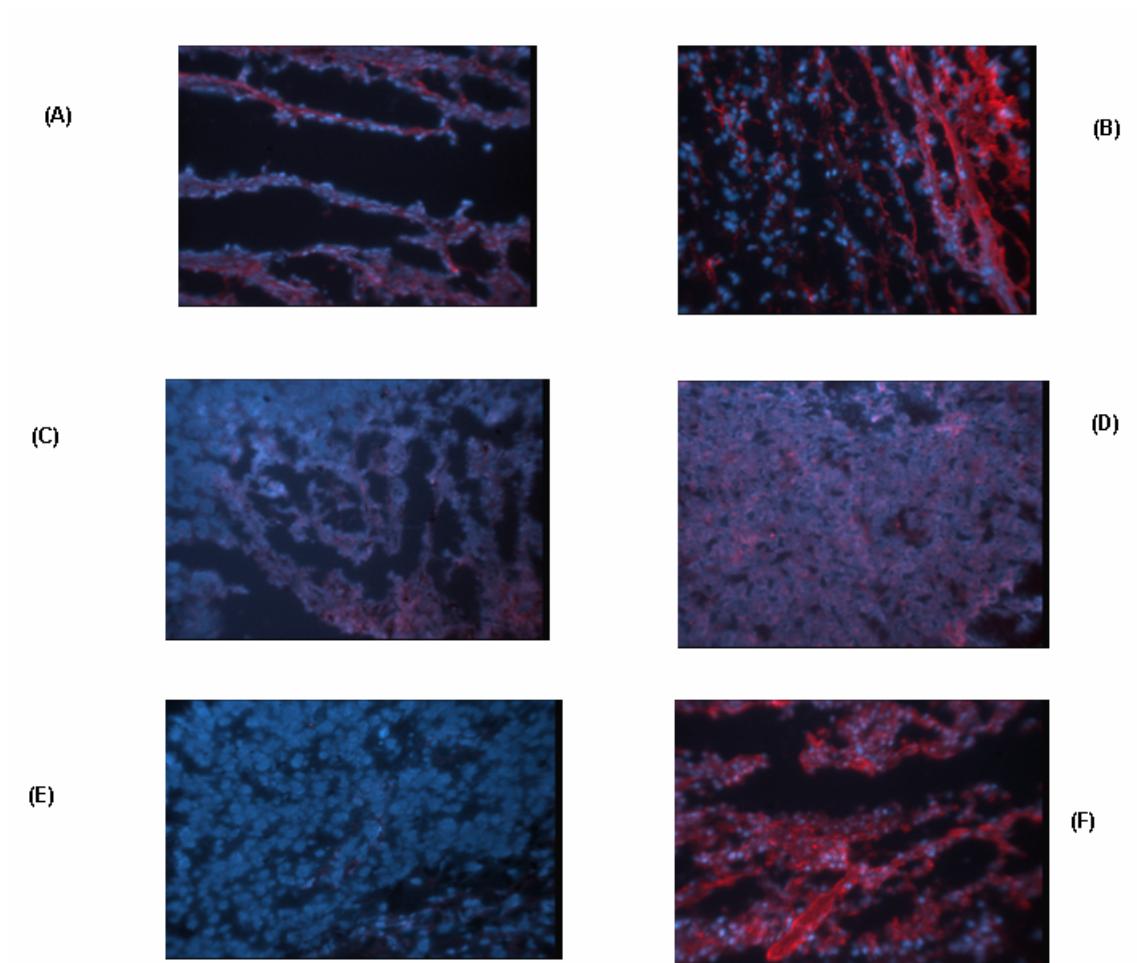


Figure 1. B16.F10 Intratumoral Immunohistochemical Staining with Vpr Antibody on Day 2 Post-Treatment. All Fields Examined at 20X Magnification. (A) P-E+ Saline 1500 V/cm, 100 μ s; (B) P+E- 100 μ g pVpr; (C) P+E+ 100 μ g pVpr 100 V/cm, 150 ms; (D) P+E+ 100 μ g pVpr 200 V/cm, 20 ms; (E) P+E+ 100 μ g pVpr 800 V/cm, 5 ms; (F) P+E+ 100 μ g pVpr 1500 V/cm, 100 μ s. Representative Tumor of Four Tumors.

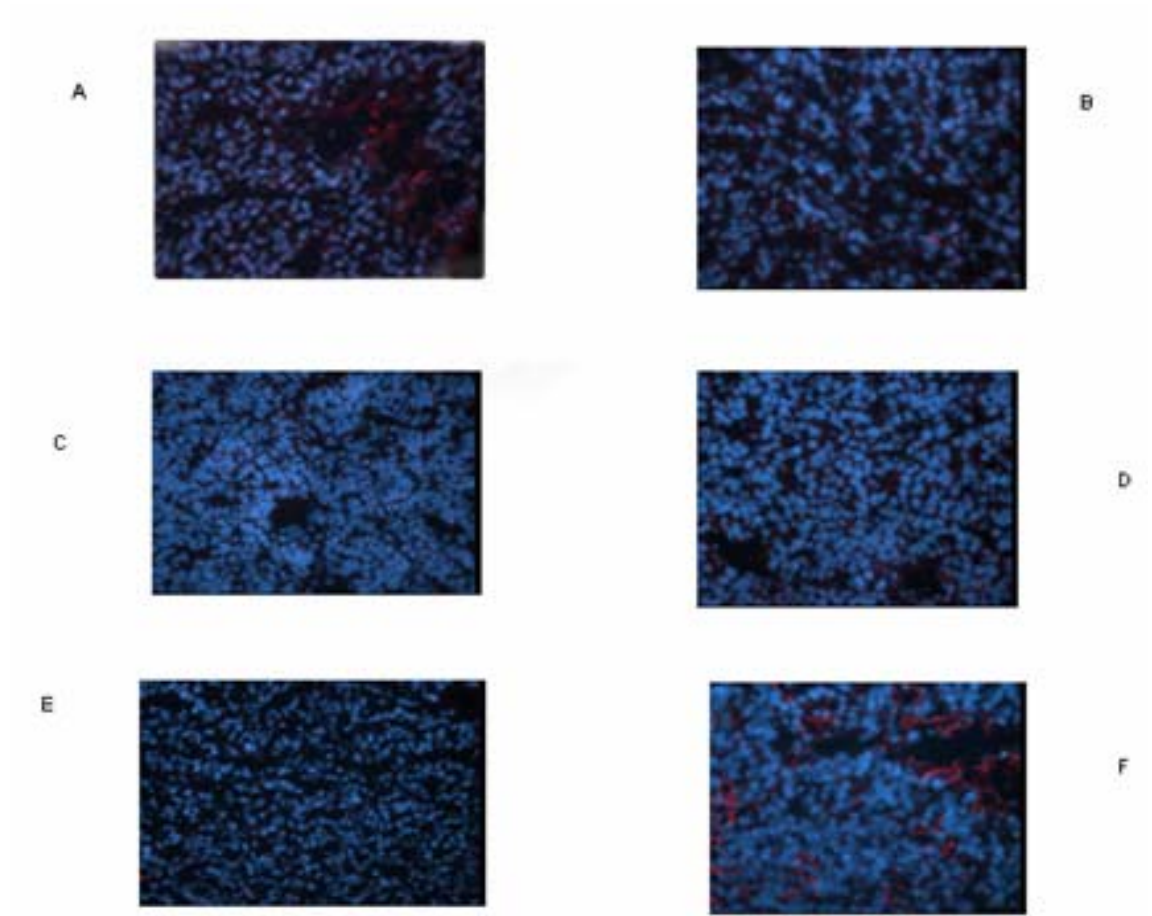


Figure 2. B16.F10 Intratumoral Immunohistochemical Staining with Vpr antibody on Day 4 Post-Treatment. All Fields Examined at 20X Magnification. (A) P-E+ Saline 1500 V/cm, 100 μ s; (B) P+E- 100 μ g pVpr; (C) P+E+ 100 μ g pVpr 100 V/cm, 150 ms; (D) P+E+ 100 μ g pVpr 200 V/cm, 20 ms; (E) P+E+ 100 μ g pVpr 800 V/cm, 5 ms; (F) P+E+ 100 μ g pVpr 1500 V/cm, 100 μ s. Representative Tumor of Four Tumors.

The electroporation parameter 1500 V/cm, 100 μ s was chosen as the best electroporation parameter because it yielded the highest protein expression at day 2 post-treatment. Therefore, 1500 V/cm, 100 μ s was used in order to determine the appropriate pVpr dosage that would cause tumor reduction after delivery of pVpr with electroporation.

B16 Growth Inhibition Following pVpr plus Electroporation Treatments on Day 0 and Day 4

Initial studies were conducted to examine tumor reduction following the selected regimen of treatment on day 0 and day 4. In the context of day 0 and day 4 treatments, it was necessary to determine which pVpr dosage would be the most efficacious in reducing B16 tumor volume. Tumor-bearing mice were treated with pVpr dosages ranging from 25 μ g to 200 μ g administered with or without electroporation. Treatments were administered on Day 0 and Day 4. The mice in the electroporation groups received 1500 V/cm, 100 μ s. The groups were as follows: Group 1: No Treatment; Group 2: P+E- 25 μ g pVpr; Group 3: P+E+ 25 μ g pVpr; Group 4: P+E- 50 μ g pVpr; Group 5: P+E+ 50 μ g pVpr; Group 6: P+E- 100 μ g pVpr; Group 7: P+E+ 100 μ g pVpr; Group 8: P+E- 200 μ g pVpr; Group 9: P+E+ 200 μ g pVpr; Group 10: V+E- 200 μ g pcDNA3.1; and Group 11: V+E+ 200 μ g pcDNA3.1. The mean tumor volumes for the groups ranged from 32-54 mm³.

The mean fold increase values for pVpr +E+ treated tumors were reduced in comparison to some of the other treatment groups (Figure 3A and Figure 3B). Day 15 is the end point for the mean fold increase curve because this is the last time point at which

a majority of untreated mice were alive for analysis. The mean fold increase values were 27.44, 17.81, 12.67, 27.81, and 9.01 for mice treated with P+E- 25 µg pVpr, P+E- 50 µg pVpr, P+E- 100 µg pVpr, P+E- 200 µg pVpr, and V+E- 200 µg pcDNA3.1, respectively (Figure 3A). The mean fold increase value for untreated mice was 24.58. The mean fold increase values for mice treated with P+E+ 25 µg pVpr, P+E+ 50 µg pVpr, P+E+ 100 µg pVpr, P+E+ 200 µg pVpr, and V+E+ 200 µg pcDNA3.1 were 16.63, 9.36, 7.89, 10.05, and 11.99, respectively (Figure 3B). By day 15, the group that received P+E+ 100 µg pVpr had the lowest mean fold increase value (i.e. 7.89) when compared to untreated mice as well as all other treatment groups. At day 15 post-treatment, the mice treated with 100 µg pVpr plus electroporation had a significant reduction in tumor volume ($p < 0.05$) in comparison to untreated mice and mice treated with 25 µg pVpr without electroporation. A significant difference in tumor reduction could not be established between any of the electroporation groups.

Even though a difference in treatment efficacy could not be established between the electroporation groups treated with various dosages of pVpr+ E+ (25 µg to 200 µg pVpr) or pcDNA+E+ (200 µg), the group that received 100 µg pVpr +E+ was the only group that had significant tumor reduction compared to untreated mice. Therefore, the 100 µg pVpr dosage with electroporation was further evaluated in the following growth inhibition studies in order to enhance the growth inhibitory effect.

As a follow-up to the initial study in which Vpr protein expression was examined after utilizing various electroporation parameters, electroporation parameters were further evaluated for the ability to induce tumor growth attenuation. Treatments were conducted

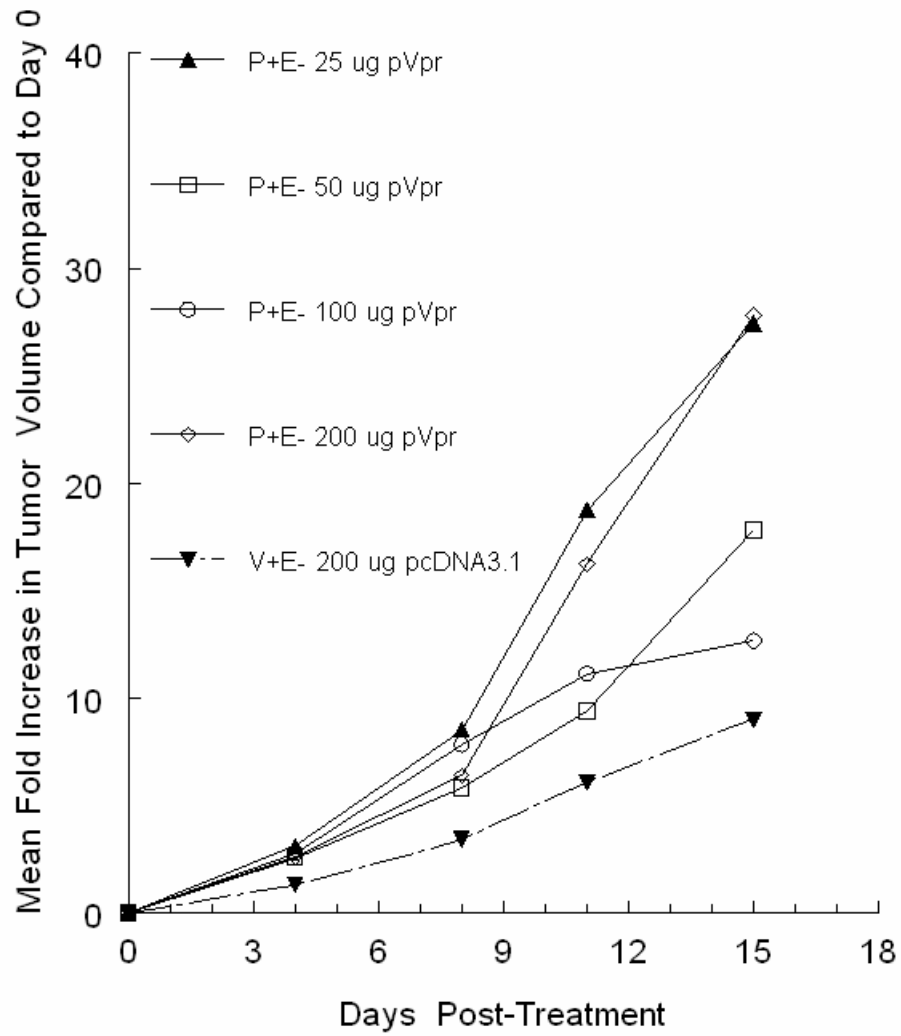


Figure 3A. Various pVpr Dosages Administered without Electroporation to B16 Melanoma

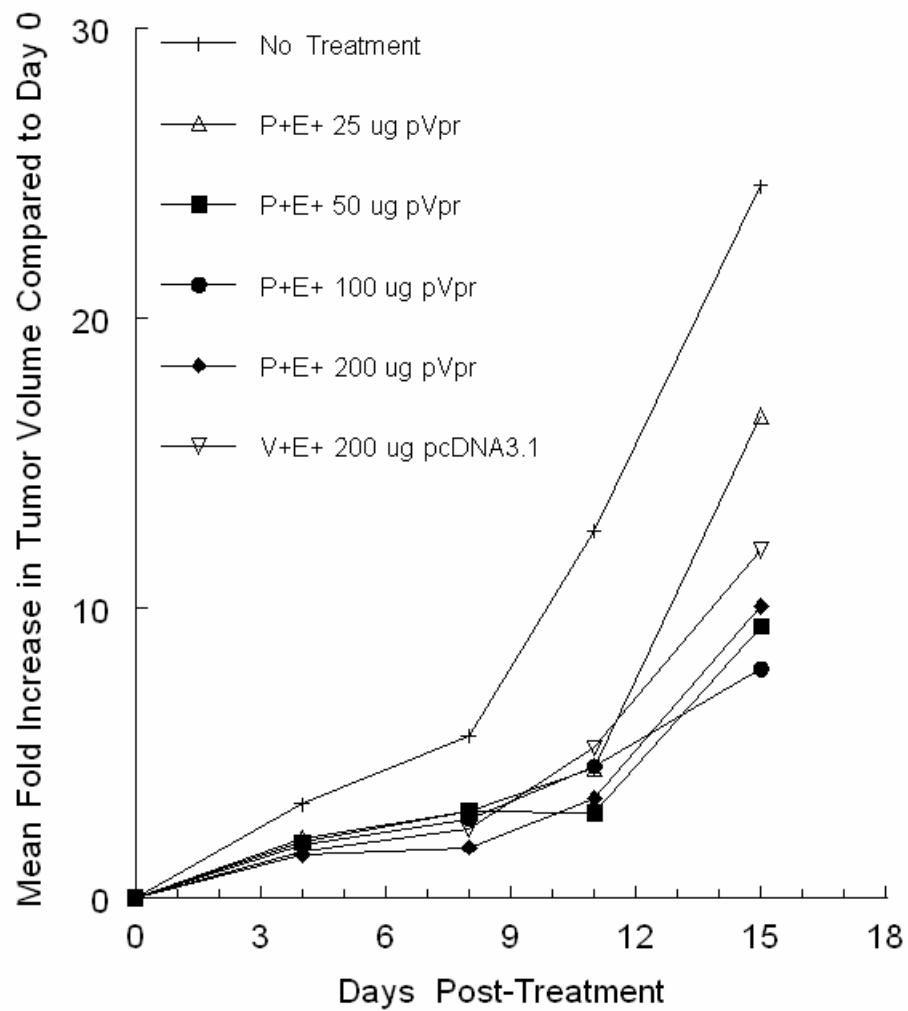


Figure 3B. Various pVpr Dosages Administered with Electroporation to B16 Melanoma

on day 0 and day 4. Average tumor volumes ranged from 29-47 cubic millimeters. There were six mice per group. The groups were as follows: Group 1: P-E+ saline with 1500 V/cm, 100 μ s (electric pulses only); Group 2: P+E- 100 μ g pVpr; Group 3: P+E+ 100 μ g pVpr 100 V/cm, 150 ms; Group 4: P+E+ 100 μ g pVpr 200 V/cm, 20 ms; Group 5: P+E+ 100 μ g 800 V/cm, 375 μ s; Group 6: P+E+ 100 μ g pVpr 1500 V/cm, 100 μ s. The mice that were treated with only electric pulses had a mean survival time of 14.33 days. Mice that were treated with 100 μ g pVpr plus 1500 V/cm, 100 μ s had a mean survival time of 18.17 days. The group that received 100 μ g pVpr plus 1500 V/cm, 100 μ s lived significantly ($p < 0.05$) longer than mice given electric pulses only and mice that received 100 μ g pVpr without electroporation (Figure 4). The remaining treatment groups had mean survival times of 14, 15, and 15.50 days for 100 V/cm, 150 ms; 200 V/cm, 20 ms; and 800 V/cm, 375 μ s, respectively.

At the early time point of day 8, the 100 μ g pVpr plus 1500 V/cm, 100 μ s treated tumors had the lowest tumor volumes compared to tumors treated with 100 μ g pVpr without electroporation and all the other 100 μ g pVpr with electroporation groups (Figure 5). The tumors treated with 100 μ g pVpr without electroporation had lower tumor volumes than all the other 100 μ g pVpr with electroporation groups at this early time point. By day 15 post-treatment the mean fold increase values for the pVpr plus 1500 V/cm group were similar to the other pVpr plus electroporation conditions.

In terms of increasing survival time for tumor-bearing mice, 100 μ g of pVpr delivered with 1500 V/cm, 100 μ s 6 pulses of electroporation worked better than its counterpart without electroporation and electric pulses alone. In fact, there was enhanced

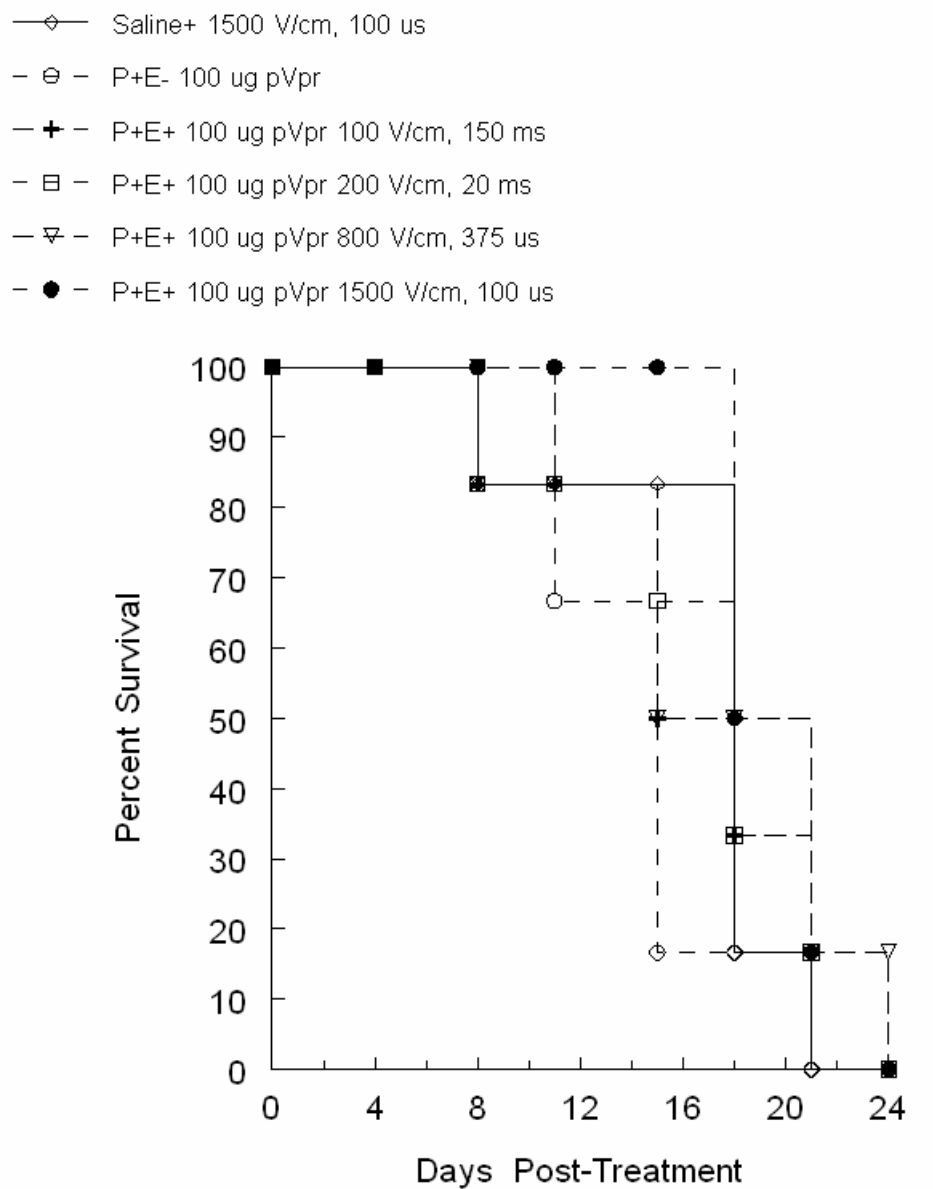


Figure 4. Percent Survival Curve for Tumor-Bearing Mice Treated with pVpr plus Various Electroporation Parameters

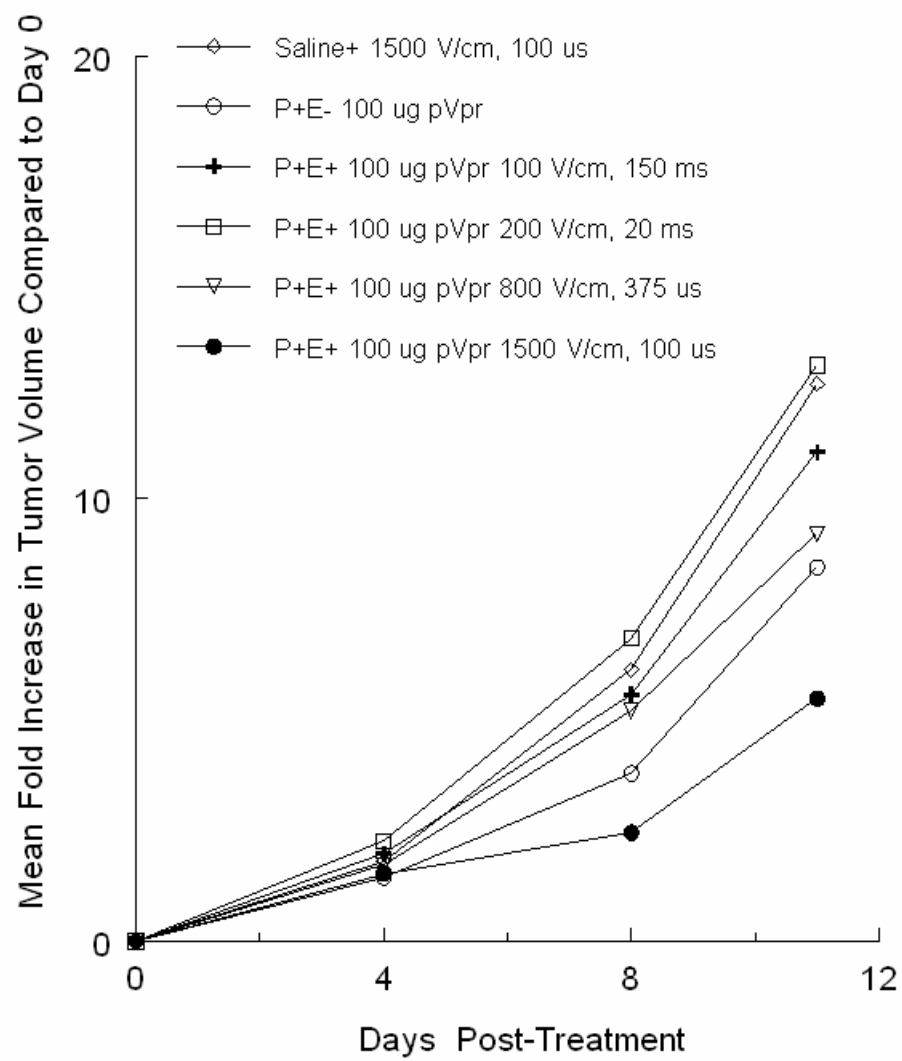


Figure 5. B16 Tumor Growth Responses to pVpr Delivered with Various Electroporation Parameters

gene expression in tumors treated with 100 µg pVpr plus 1500 V/cm in comparison to tumors treated with 100 µg pVpr without electroporation. At the early time points the other electroporation conditions offered the same tumor growth inhibition as the electric pulses alone (1500 V/cm, 100 µs). Taken together with the immunohistochemical staining, this data suggests that the other electroporation conditions did not provide gene expression perhaps because these conditions did not overcome the threshold level that would allow for efficient gene delivery. Therefore, the other electroporation parameters did not provide enough pVpr for efficient Vpr expression to slow tumor growth.

The 1500 V/cm electroporation condition had the greatest impact on tumor reduction from the onset of the experiment to day 15 post-treatment. It took 15 to 18 days for 100 µg of pVpr plus 1500 V/cm treated tumors to grow to the same size as the tumors in the other pVpr plus electroporation conditions. Therefore, it would be best to use 1500 V/cm, 100 µs as the electroporation parameter for future experiments to obtain efficient gene expression and apoptosis.

To further evaluate and confirm the growth inhibition following delivery of pVpr with the 1500 V/cm, 100 µs pulsing conditions, another experiment was performed. Treatments were administered on Day 0 and Day 4. Initial tumor volumes ranged from 37 – 42 cubic millimeters. There was an n=7 for each group. The groups were as follows: no treatment; 25 µg pVpr+E- ; 25 µg pVpr+E+; 100 µg pVpr+E-; 100 µg pVpr+E+; 100 µg pcDNA3.1+E-; 100 µg pcDNA3.1+E+; and saline+ E+. By day 7 post-treatment, the mice treated with either dosage of pVpr+E+ or pcDNA+E+ had lower tumor volumes than all the other treatment groups including the saline+E+ group. Day 21 was chosen as

the end point for tumor growth analysis because this is the last day at which untreated mice were still alive for inclusion in the analysis. The untreated tumors grew so aggressively that all these mice were overcome by tumor burden. By day 21 post-treatment, the mean fold increase value was 12.21 for the 100 μ g pVpr+E+ group. In contrast, on day 21, the mean fold increase value was 34.98, 31.38, 45.20, 18.24, and 72.60 for the groups 25 μ g pVpr+E-, 25 μ g pVpr+E+, 100 μ g pVpr+E-, 100 μ g pcDNA3.1+E+, and untreated mice, respectively (Figure 6). The 100 μ g pcDNA3.1+E- group and the saline +E+ group were dead after day 18 and were not included in this analysis. Therefore, by day 21, the 100 μ g pVpr+E+ treatment group had the largest reduction in tumor volume in comparison to the other treatment groups.

Even though the 100 μ g pVpr+E+ treated mice had the largest reduction in tumor volume during the earlier time points, these tumor reductions did not result in high percentages of long-term complete tumor regressions at latter time points. Of the mice that were previously treated with 100 μ g pVpr+E+, 1 out of 7 mice had complete tumor regression for over 100 days. Interestingly, 2 out of 7 mice treated with 100 μ g pcDNA3.1+E+ also had complete tumor regression for over 100 days. This type of long-term regression has been previously reported by our group after mice were treated with a non-coding vector plus electroporation conditions (800 V/cm, 5 ms, 10 pulses) other than the ones used in the current experiment. However, this preliminary data demonstrated that intratumoral delivery of 100 μ g pVpr consistently slowed B16 tumor growth and resulted in a long-term complete tumor regression. The results of this preliminary experiment warranted further investigation of the influence of 25 μ g pVpr+E+, 100 μ g

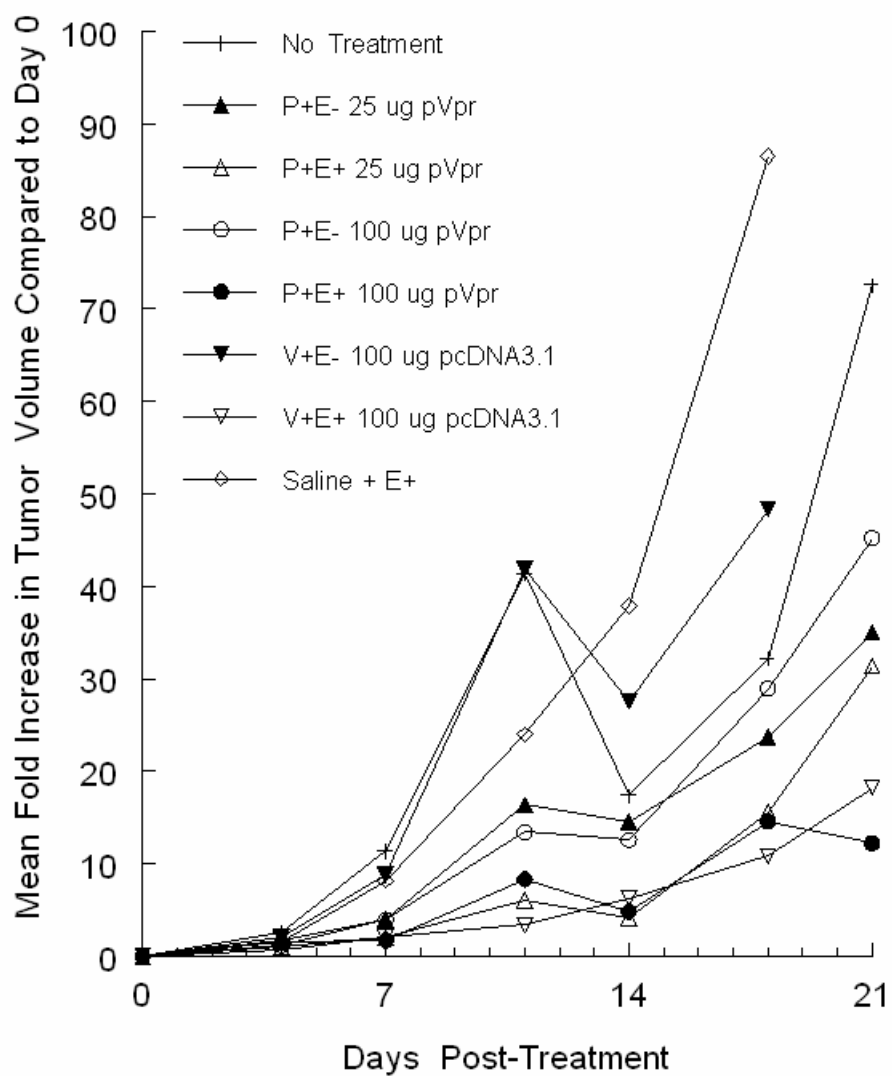


Figure 6. B16 Tumor Growth Inhibition Following Treatment with pVpr plus 1500 V/cm, 100 μ s

pVpr+E+, and 100 µg pcDNA3.1 +E+ on B16 growth and regression after day 0 and day 4 treatment. In addition, the anti-proliferative effects of Vpr protein warranted further investigation into the optimization of the frequency of treatment days.

Treatment with pVpr plus Electroporation on Four Treatment Days

The anti-proliferative effects of Vpr protein were further investigated by increasing the frequency of treatment days as well as re-evaluating the range of pVpr dosages that would lead to tumor growth inhibition in the context of these additional treatment days. To maximize B16 tumor reduction, an experiment was conducted to treat tumor-bearing mice with various doses of pVpr ranging from 25 µg to 100 µg pVpr administered with or without electroporation on four treatment days (i.e. days 0, 4, 7, & 11). The appropriate non-coding control plasmid with electroporation, pcDNA3.1, was used at the 100 µg concentration. There were seven mice per group. The electroporation parameters were 1500 V/cm, 100 µs. The treatment groups were as follows: Group 1: No treatment; Group 2: P+E- 25 µg pVpr; Group 3: P+E+ 25 µg pVpr; Group 4: P+E- 50 µg pVpr; Group 5: P+E+ 50 µg pVpr; Group 6: P+E- 100 µg pVpr; Group 7: P+E+ 100 µg pVpr; Group 8: V+E- 100 µg pcDNA3.1; Group 9: V+E+ 100 µg pcDNA3.1.

Since there were no complete tumor regressions or partial tumor regressions in this experiment, the mean tumor doubling time, mean fold increase in tumor volume, and the mean survival time were determined. The untreated mice had a mean tumor doubling time of 7.67 days. The mice that received 25 µg pVpr or 50 µg pVpr without electroporation had mean tumor doubling times of 6.71 and 6.67 days, respectively. The mice that received 100 µg pVpr without electroporation and 100 µg pcDNA3.1 without

electroporation had mean tumor doubling times of 8.14 and 9.86 days, respectively. The mice that were exposed to 50 µg pVpr with electroporation had a mean tumor doubling time of 13.71 days. The mice that were exposed to 100 µg pcDNA3.1 plus electroporation had a mean tumor doubling time of 13.14 days. The mice treated with 100 µg pVpr plus electroporation had a mean tumor doubling time of 12.83 days.

Mice that received pVpr or pcDNA3.1 plus electroporation had a reduction in tumor volume. Day 15 post-treatment is the end point for the mean fold increase curve because this was the last time point at which more than 40% of untreated mice could be included for accurate analysis. At day 15, the untreated mice had a mean fold increase of 15.71. At day 15, the mean fold increase value for mice treated with P+E- 25 µg pVpr, P+E- 50 µg pVpr, P+E- 100 µg pVpr, and V+E- 100 µg pcDNA3.1 was 13.08, 20.19, 18.48, and 15.46, respectively. Also at day 15, the mean fold increase value for mice treated with P+E+ 25 µg pVpr, P+E+ 50 µg pVpr, P+E+ 100 µg pVpr, and V+E+ 100 µg pcDNA3.1 was 5.18, 4.35, 4.75, and 5.14, respectively (Figure 7).

Some of the treatment regimens that incorporated electroporation had an impact on mean survival time of tumor-bearing mice. Untreated mice had a mean survival time of 14.14 days. Mice treated with P+E- 25 µg pVpr, P+E- 50 µg pVpr, P+E- 100 µg pVpr, and V+E- 100 µg pcDNA3.1 had mean survival times of 16, 13.71, 18.86, and 17.43, respectively. Mice treated with P+E+ 25 µg pVpr, P+E+ 50 µg pVpr, P+E+ 100 µg pVpr, and V+E+ 100 µg had mean survival times of 18.71, 25.86, 21.57, and 20.29. Mice that were treated with P+E+ 50 µg pVpr and P+E+ 100 µg pVpr were the only groups that lived significantly ($p < 0.05$) longer than untreated mice.

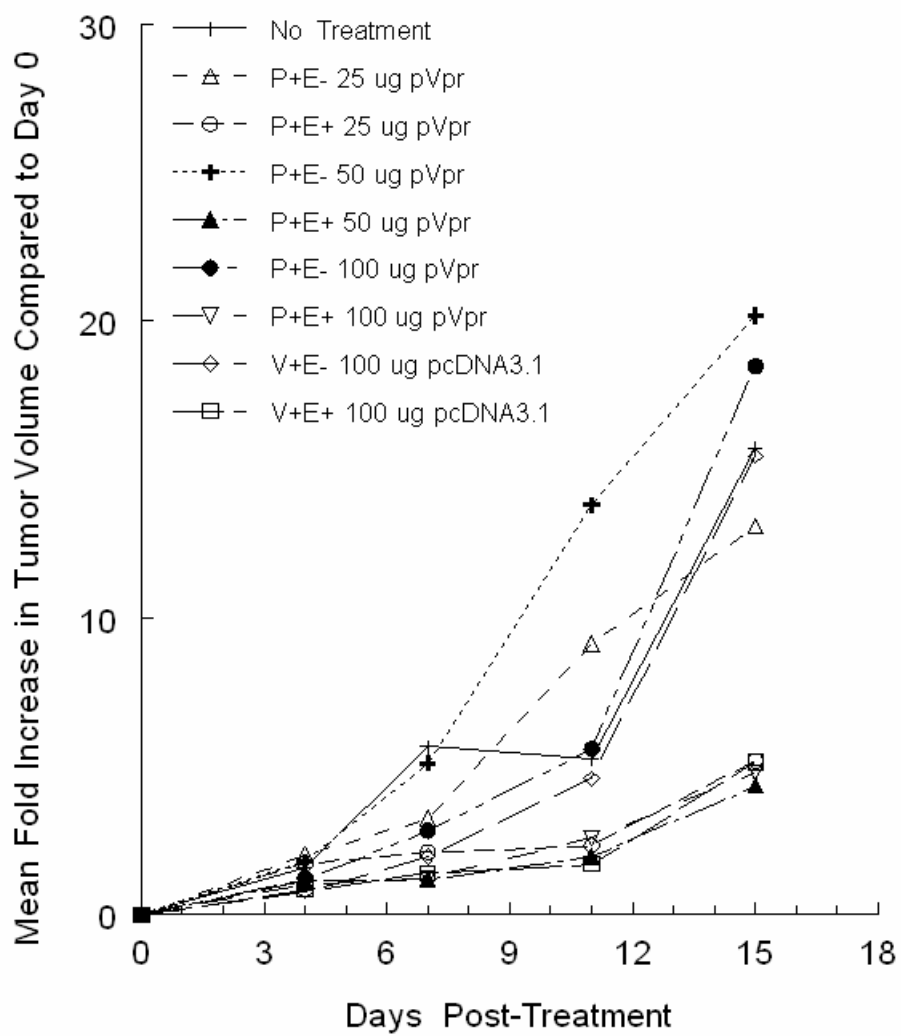


Figure 7. B16 Tumor Growth Attenuation Following Various Dosages of pVpr plus Electroporation

Consecutive Treatment Days versus Alternating Treatment Days with pVpr plus Electroporation

Since subcutaneous B16 tumors grow so aggressively, it was necessary to have treatments occur at closer intervals to combat tumor growth that would occur between the treatment days that were previously mentioned such as treatment days 0, 4, 7, and 11. As part of the evaluation of the anti-proliferative effects of Vpr protein, pVpr plus electroporation treatments were administered to tumor-bearing mice to determine whether consecutive treatment days or alternating treatment days would lead to enhanced tumor growth inhibition. Mice were treated with 100 µg pVpr with 1500 V/cm, 100 µs. The consecutive treatment days were days 0 and 1. The alternating treatment days were 0 and 2. There was no difference in mean tumor doubling time since both treatment regimens resulted in a mean tumor doubling time of 8 days. The mean fold increase curve illustrates the first 11 days because this is the last time point at which the majority of mice were still alive to obtain an accurate assessment of statistical analysis. In accordance with the mean fold increase data, at the early time points, the consecutive treatment regimen caused more reduction in tumor volume than the alternating treatment regimen. However, by day 8 and 11 post-treatment, the alternating treatment appears more effective in reducing tumor volume (Figure 8). The preliminary experiments which examined the effects of delivering pVpr with electroporation on alternating treatment days resulted in tumor growth inhibition.

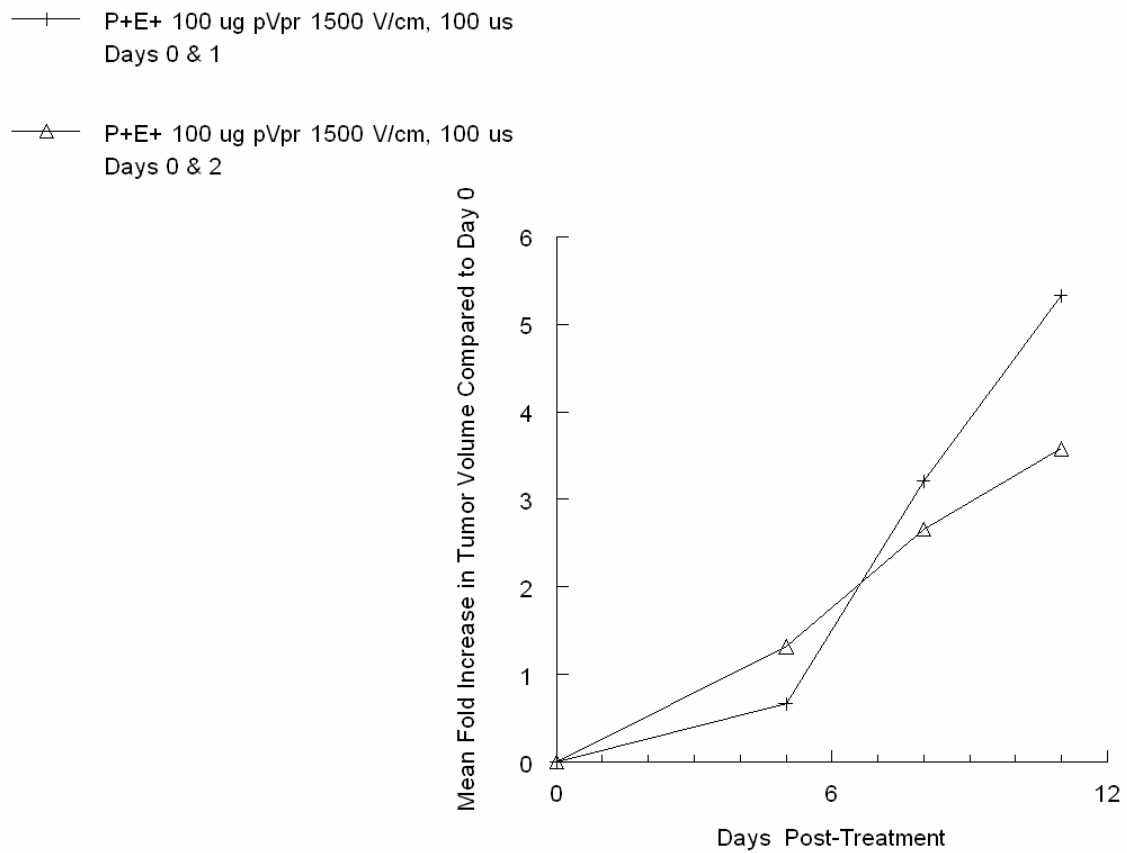


Figure 8. Growth Attenuation in B16 Tumor Volume Following Treatment with pVpr plus Electroporation on Alternating Treatment Days

Therapeutic Benefit of Daily Treatment with pVpr plus Electroporation

In accordance with the data demonstrating the beneficial aspects of tumor reduction following a consecutive treatment regimen with pVpr plus electroporation, it was necessary to determine whether there would be additional benefits in extending the consecutive treatment regimen to include more days. A combination of experiments were conducted to determine whether daily treatment (i.e. day 0- day 7) would lead to enhanced tumor reduction and regression after tumor-bearing mice were exposed to 100 µg pVpr plus electroporation. However, this experiment had additional treatment groups which varied in the days and frequency of treatment administration including the original treatment regimen of day 0 and day 4. The results represent a combination of two experiments that were conducted to give a total n of five mice per group with the exception of the untreated mice and the pcDNA +E+ treated mice. The 100 µg pcDNA3.1 + E+ group was included in the second experiment as a control group and had an end-point of 62 days. The treatment groups were as follows: Group 1: P-E+ 1500 V/cm, 100 µs saline twice daily (treatment days 0-7); Group 2: P+E+ 100 µg pVpr 1500 V/cm, 100 µs twice daily (treatment days 0-7); Group 3: P+E+ 100 µg pVpr 1500 V/cm, 100 µs once daily (treatment days 0-7); Group 4: P+E+ 100 µg pVpr 1500 V/cm, 100 µs on days 0, 2, 4, & 6; Group 5: P+E+ 100 µg pVpr 1500 V/cm, 100 µs on days 0 & 4; and Group 6: No treatment (n=3); and Group 7: V+E+ 100 µg pcDNA3.1 1500 V/cm, 100 µs once daily (n= 4).

The various pVpr +E+ treatment regimens extended the lifespan of treated mice. The untreated tumors grew very aggressively. The mean survival time for untreated mice

was 11.67 days. Mice treated with 100 µg pVpr plus electroporation twice daily and once daily had a mean survival time of 146.20 and 122, respectively. Mice treated with 100 µg pVpr +E+ on days 0 & 4 and mice treated with 100 µg pVpr +E+ on days 0, 2, 4, & 6 had mean survival times of 28 and 55.60 days, respectively. All of the mice treated once daily with 100 µg pcDNA3.1 +E+ had a mean survival time of 62 days.

Mice treated with electric pulses administered twice daily had a mean survival time of 26.80 days. Mice treated with 100 µg pVpr +E+ twice daily lived significantly longer ($p<0.05$) than mice treated with 100 µg pVpr +E+ on days 0, 2, 4, & 6; 100 µg pVpr +E+ on days 0 & 4; saline +electric pulses twice daily; and untreated mice. Mice treated with 100 µg pVpr +E+ once daily lived significantly longer ($p<0.05$) than mice treated with 100 µg pVpr +E+ on days 0 & 4; saline +electric pulses twice daily; and untreated mice. Mice treated with 100 µg pVpr +E+ on days 0 & 4 lived significantly longer ($p<0.05$) than untreated mice. There is no statistical difference between the mean survival times for the mice treated with pVpr +E+ twice daily or once daily.

Day 21 post-treatment is the end-point for the mean fold increase curve because this is the last time at which a majority of the saline plus electroporation treated mice were alive. At day 21 post-treatment, the mice that were treated with 100 µg pVpr plus electroporation either twice daily or once daily had significant tumor reduction ($p<0.05$) in comparison to mice treated with only electric pulses twice daily (Figure 9). There is no difference between any of the other pVpr+ E+ treatment groups in terms of mean fold increase values. However, there are differences in pVpr +E+ treatment regimens in terms of complete tumor regressions. One hundred days after initial treatment, 80% of mice

receiving either once daily or twice daily intratumoral delivery of 100 µg pVpr with electroporation were tumor-free. These mice were considered cured. Following intratumoral delivery of pVpr plus electroporation on Days 0, 2, 4 & 6, one tumor-bearing mouse was cured. Tumor volume continued to decrease until all four of the pcDNA3.1+ E+ treated mice had complete regressions by day 18 post-treatment (Figure 9). The daily pVpr +E+ treatment regimens were more effective than the original pVpr + E+ treatment regimen of day 0 & day 4 in terms of increasing the longevity of mice as well as inducing a higher percentage of complete regressions.

Intratumoral pVpr plus Electroporation Administered with a Revised Alternating Treatment Regimen

Daily treatments offered 80 – 100% complete tumor regressions irrespective of pVpr + E+ or pcDNA +E+ treatment. Therefore, it was necessary to devise an alternative treatment regimen that would offer tumor growth inhibitions, complete tumor regressions, and distinction between pVpr and pcDNA effects. Since the original alternating pVpr plus electroporation treatment administered on days 0 & 2 yielded growth inhibition, the concept of administering pVpr with electroporation on alternating treatment days was revisited with an additional treatment day included. Mice were treated with 100 µg pVpr plus electroporation on Days 0, 2, and 4. 100 µg pcDNA3.1 plus electroporation was used as a control. The electroporation parameters were 1500 V/cm, 100 µs. Each group had an n=3. The mice treated with 100 µg pVpr plus electroporation experienced both a partial response and complete response towards the treatment. From days 9 through 16 post-treatment, one mouse treated with pVpr plus electroporation had complete tumor regression until the tumor recurred. There was a slight pcDNA +E+

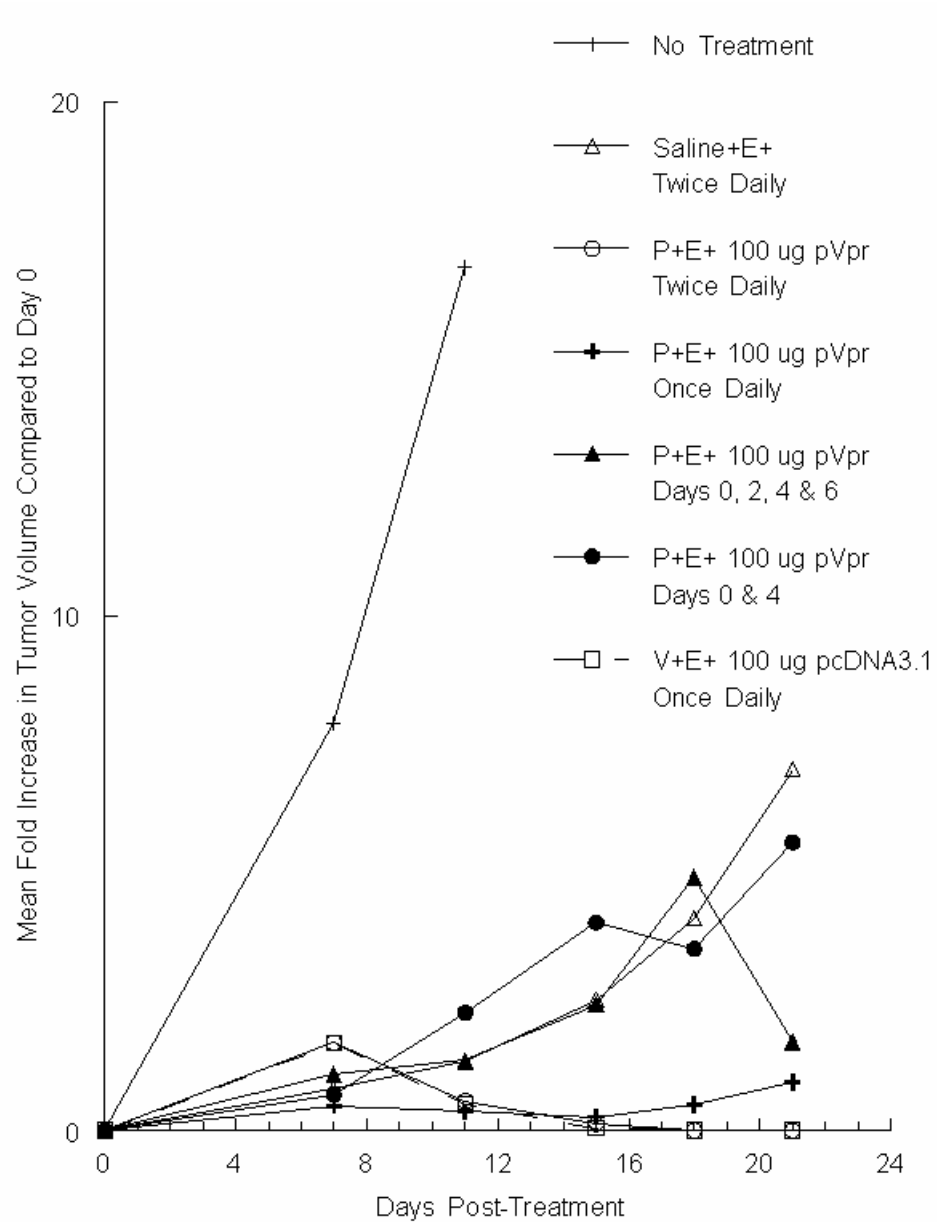


Figure 9. Daily Treatment with 100 μ g pVpr plus Electroporation Leads to Enhanced Tumor Reduction

effect observed at later time points as one pcDNA +E+ treated mouse had complete tumor regression for 18 days. The mean fold increase curve ends at day 24 because this is the last time point at which at least two mice are still alive in each group. In comparison to the pcDNA+E+ treated mice, the mice treated with 100 µg pVpr plus electroporation had notable growth reduction over the course of the experiment especially from days 9 through 16 post-treatment (Figure 10). There was no difference in the mean survival time since the mean survival time was 29.0 and 27.3 days for pVpr+ E+ and pcDNA3.1+ E+, respectively.

Another experiment was conducted to further investigate the initial effect of three treatments with pVpr+ E+ in the context of the proper control groups. This experiment had an n=4 mice per group. Treatments were conducted on days 0, 2, and 4. The electroporation parameters were 1500 V/cm, 100 µs. The treatment groups were as follows: no treatment; 100 µg pVpr+E-; 100 µg pVpr+E+; 100 µg pcDNA3.1+E-; 100 µg pcDNA3.1+E+; and saline +E+ (electric pulses alone). The 100 µg pVpr+E+ group had the most tumor reduction compared to the other treatment groups. In fact, the B16 tumors grew so aggressively that by day 17 post-treatment, all the groups except for the untreated group and the 100 µg pVpr+E- group had enough mice still alive for meaningful statistical analysis. However, mice in the saline+E+ group were dead after day 17 post-treatment. Therefore, Day 17 was chosen as the end point for the mean fold increase analysis. The mean fold increase values were 2.17, 46.45, 4.46, and 10.44 for the group treated with 100 µg pVpr+E+, 100 µg pcDNA+E-, 100 µg pcDNA+E+, and saline+ E+, respectively (Figure 11). By day 24, one 100 µg pcDNA3.1+ E+ treated mouse experienced a complete tumor regression that lasted past 100 days post-treatment.

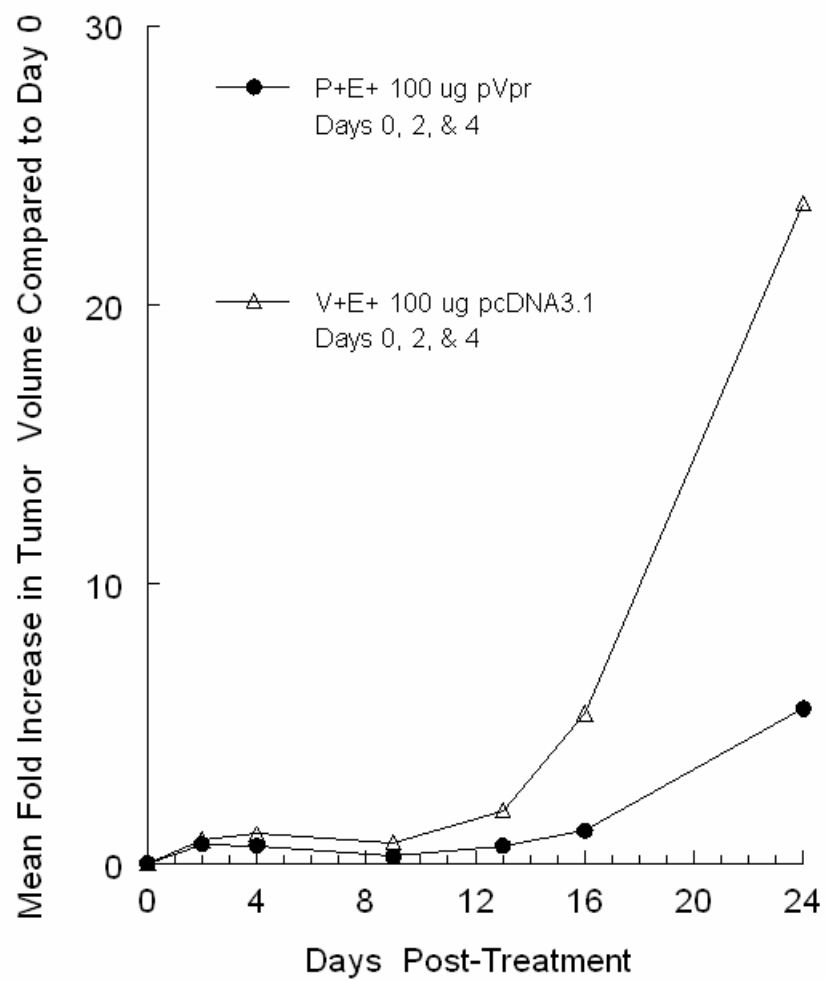


Figure 10. A Three Treatment Day Regimen Leads to Tumor Volume Reduction

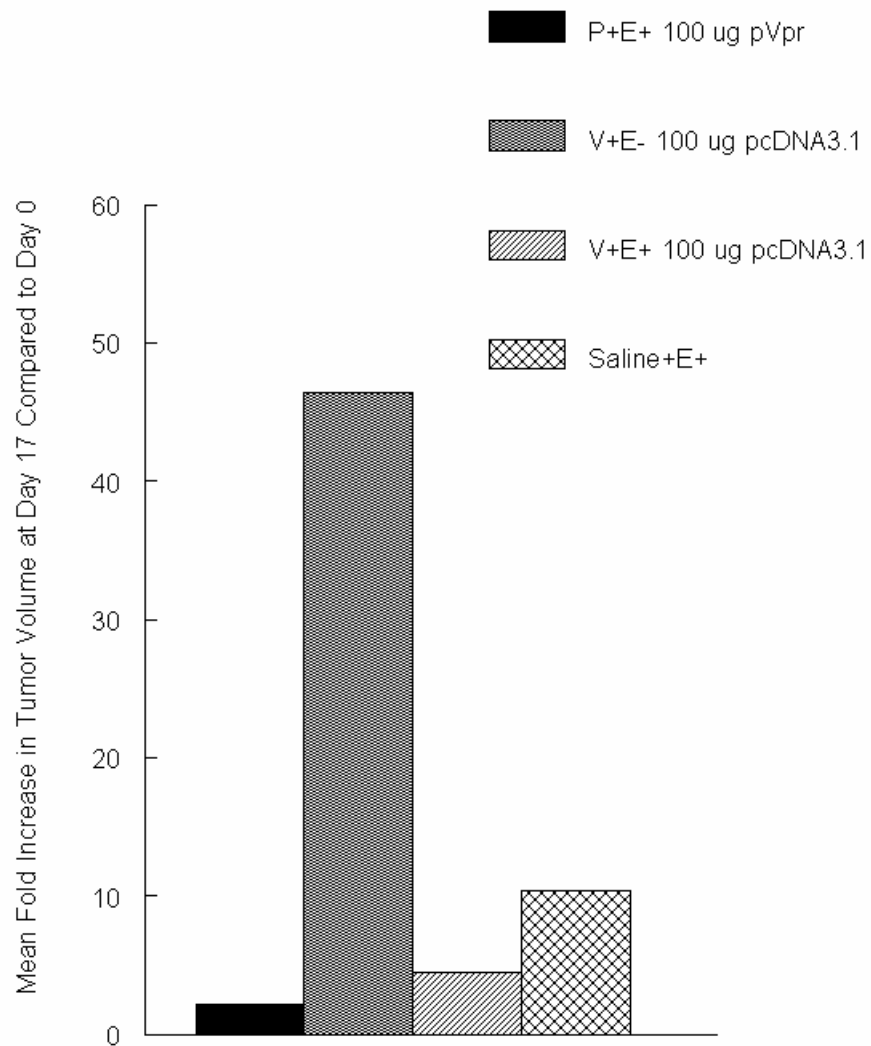


Figure 11. Three Treatments with 100 μ g pVpr Causes Tumor Growth Delay Three Weeks After Treatment

Optimal pVpr Treatment Regimen

Optimal pVpr treatment is defined as the treatment regimen that not only yields tumor growth inhibition but long-term complete tumor regressions as well. Two protocols from the preliminary experiments yielded either short-term or long-term regressions. These regimens were further examined as optimal pVpr treatment regimens.

Complete Regression of Established Subcutaneous B16 Tumors After Treatment with pVpr plus Electroporation

To determine whether there was a therapeutic effect following the delivery of pVpr with electroporation, post-treatment tumor volume measurements were made to assess any attenuation of tumor growth as well as the induction of complete tumor regressions coupled with long-term survival. The average tumor volumes for each group ranged from 38 – 74 mm³. The electroporation parameters were 1500 V/cm, 100 μ s. The tumors were treated with either 25 μ g or 100 μ g of pVpr on days 0 and 4 with or without electroporation. Figure 12 summarizes melanoma tumor growth in the different treatment groups during the initial 25 days of the experiment. Day 25 post-treatment was chosen as the end-point for the mean fold increase curve because this is the last time point at which a majority of the treated mice were still alive for accurate analysis. The absence of a mean fold tumor volume increase value at day 25 for a particular group indicates that all of the mice were dead by that time point.

During the first week following treatment, tumor growth was inhibited to some extent in all the treatment groups when compared to the untreated control group

(i.e. Figures 12B, C and D versus A). Values for the mean fold increase in tumor volume, compared to day 0, were indicated for all the treatment groups through day 18 since at this point at least 50% of all of the mice in each of the treatment groups were still alive with the exception of 3 (21.4%) mice alive in the untreated group. At day 18 post-treatment the mean fold tumor volume increases were significantly decreased ($p < 0.05$) for the P+E+ 25 μ g pVpr, P+E- 100 μ g pVpr, and P+E+ 100 μ g pVpr groups with values of 11.3, 21.2 and 7.6, respectively, compared to the no treatment group (mean fold tumor volume increase of 45.4). The value for the P+E- 25 μ g pVpr group was not significantly different to that of the no treatment group. Likewise, at day 18, the V+E- 100 μ g pcDNA3.1 and V+E+ 100 μ g pcDNA3.1 groups but not the saline plus electroporation group were significantly decreased compared to the no treatment group. For the groups with mean fold tumor volume increase values at day 25, both the P+E+ 25 μ g and P+E+ 100 μ g pVpr groups had significantly decreased values (i.e. 16.2 and 12.4, respectively) compared to the V+E+ 100 μ g pcDNA3.1 group (i.e. 29) and the P+E- 100 μ g pVpr group (i.e. 74.4). These results indicated an initial backbone vector plus electroporation effect, in terms of slowing of tumor growth. However, at day 25, the mean fold tumor volume increases for the pVpr plus electroporation treatment groups were significantly suppressed compared to the values measured in the pcDNA3.1 control plus electroporation group.

While tumor reduction is an important evaluative criterion for assessing therapeutic potential, the critical parameter is the regression of existing tumors, i.e. a cure. Long-lasting tumor regressions were only observed in the groups that received the

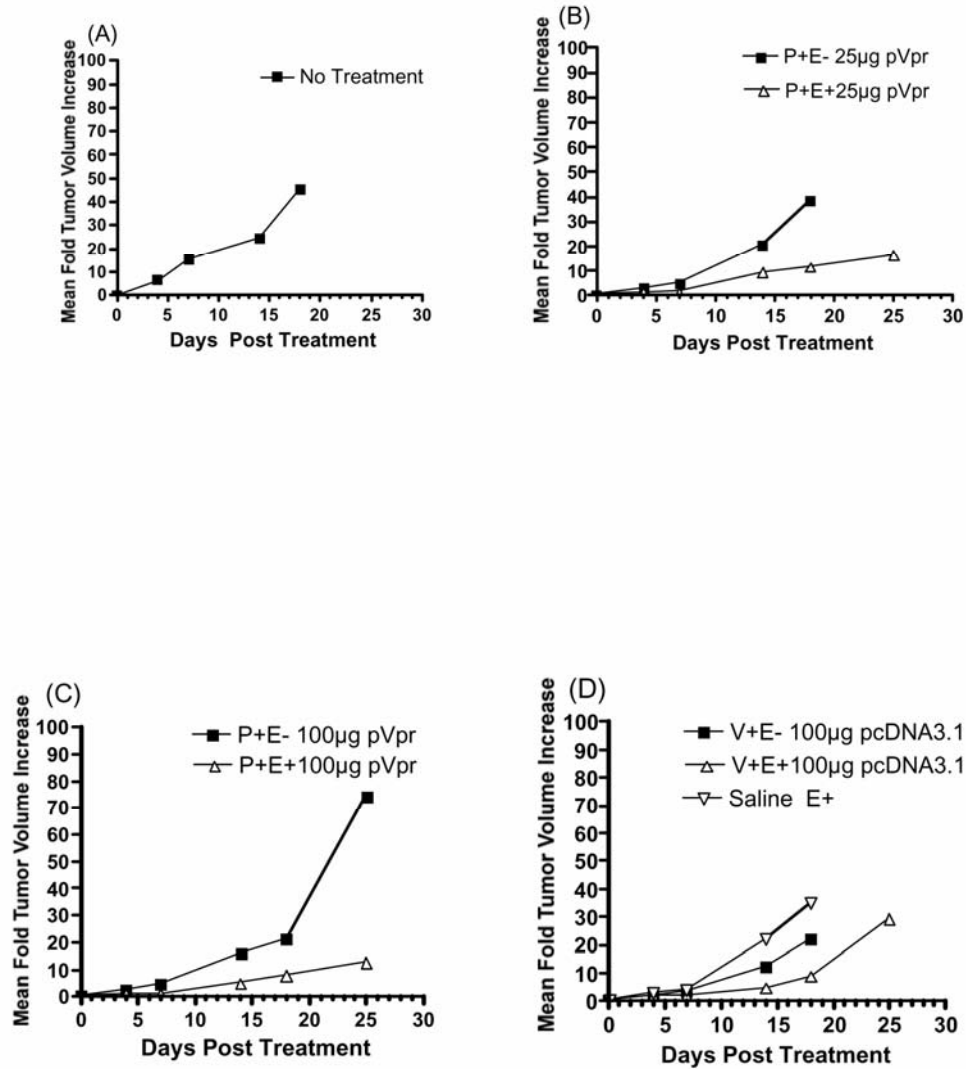


Figure 12. B16 Melanoma Tumor Growth Responses to pVpr Delivered with or without *In vivo* Electroporation. Mean Fold Increase in Tumor Volume (Compared to day 0) at Various Post-Treatment Time Points for the Different Treatment Groups: (A) No Treatment; (B) 25 µg pVpr with or without Electroporation; (C) 100 µg pVpr with or without Electroporation and (D) 100 µg pcDNA3.1 with or without Electroporation and Saline plus Electroporation. Reprinted, by permission, from Mol Ther 2006; 14:647-55

pVpr +E+ (Table 1). The 100 µg pVpr+ E+ group had the highest percentage of mice with complete tumor regressions (42.9%) on day 14 post-treatment. In addition, on day 14 post-treatment, mice that received 25 µg pVpr+ E+ or 100 µg pcDNA3.1+ E+ had complete tumor regressions in 14.3% and 7.1% of mice, respectively. It is important to note that the 100 µg pcDNA3.1 +E+ treated mouse that experienced tumor regression remained tumor free for only 4 days before recurrence of the tumor. At day 28 post-treatment, 14.3% of mice treated with 25 µg pVpr +E+ and 21.4% of mice treated with 100 µg pVpr +E+ had complete tumor regressions. It is important to indicate that none of the mice in the pcDNA3.1+ E+ treatment group underwent prolonged complete tumor regression nor did they survive long-term. This finding is presented in Figure 13.

As indicated, the ultimately relevant end-point indicating the anti-tumor efficacy of the pVpr plus electroporation treatment would be the ability of this regimen to induce complete tumor regression coupled with long-term survival of the mice, i.e. a cure. Sustained tumor regression and survival for 100 days post-treatment is considered the benchmark for cure in mice in this model. Therefore, the survival rate and complete tumor regression status past 100 days were examined in this study. These data are presented in the Kaplan-Meier survival curve in Figure 13. Mice in the untreated group as well as groups receiving 25 µg or 100 µg pVpr without electroporation, pcDNA3.1 with or without electroporation, and saline plus electroporation all succumbed to tumor burden with none surviving to the day 100 benchmark. The mean survival time for untreated mice was 13.6 days. Mice in the untreated group had a significantly shorter survival time ($p<0.05$) when compared to groups that received either dose of pVpr+E+ or pVpr+E-, pcDNA+E+ or pcDNA+E-, & saline+E+. The mean survival time for mice treated

TABLE 1. Percent of Mice with Complete Tumor Regressions

Groups	Day 14	Day 28
No Treatment	0	N/A
P+E- 25 ug pVpr	0	N/A
P+E+ 25 ug pVpr	14.29% (2 out of 14)	14.29% (2 out of 14)
P+E- 100 ug pVpr	0	N/A
P+E+ 100 ug pVpr	42.86% (6 out of 14)	21.43% (3 out of 14)
V+E- 100 ug pcDNA3.1	0	N/A
V+E+ 100 ug pcDNA3.1	7.14% (1 out of 14)*	0
Saline+E+	0	N/A

N/A indicates that all mice in this group are dead at this time point

* indicates that this mouse only had tumor regression for 4 days before tumor recurred.

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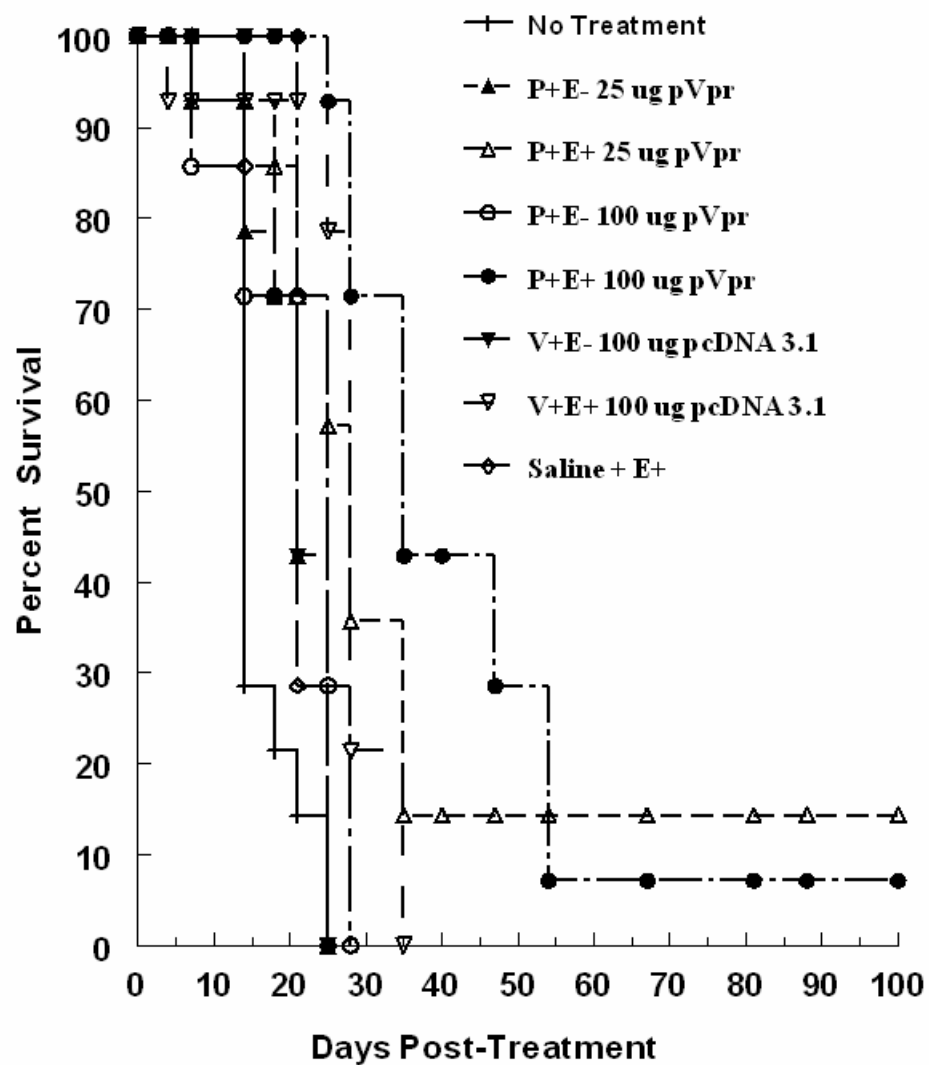


Figure 13. Kaplan-Meier Survival Curve of Mice in Different Treatment Groups up to 100 Days Post- Treatment

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with 25 or 100 μ g pVpr without electroporation, 100 μ g pcDNA without & with electroporation, or saline plus electroporation (i.e. electric pulses alone) were 17.8, 19.4, 19.3, 24.3 and 17.3 days, respectively. The mean survival times for the groups treated with 25 or 100 μ g pVpr plus electroporation were 40.5 and 43.2 days, respectively. There was no statistical difference between the mean survival time for mice treated with 25 μ g and 100 μ g pVpr plus electroporation.

Delivery of the plasmid with electroporation in the 25 μ g or 100 μ g pVpr treatment regimens resulted in complete tumor regression coupled with long-term survival at least for 100 days in 14.3% and 7.1% of the mice, respectively. Likewise, there was no statistically significant difference ($p < 0.05$) between the regression/long-term survival rates in the 25 μ g versus 100 μ g pVpr plus electroporation treatment groups.

Growth Inhibition and Regression after Multiple Treatments with pVpr plus Electroporation

Experiments were conducted to examine the anti-tumor effect of electroporation-mediated delivery of pVpr administered on multiple treatment days. 100 μ g of pVpr or pcDNA was administered with or without 1500 V/cm, 100 μ s on treatment days 0, 2, and 4. The initial mean tumor volumes ranged from 95 -122 mm³. The mean tumor volume was examined for the various groups for the first eight days after treatment. Day 8 is the last time point at which at least 50% of the mice were still alive in all the treatment groups. The mice that were treated with 100 μ g pVpr plus electroporation had tremendous tumor reduction in comparison to the other treatment groups, especially

the mice treated with 100 µg pcDNA3.1 with electroporation (Figure 14). In addition to growth attenuation, complete tumor regression was evaluated as an indication of pVpr treatment efficacy. Twelve days after exposure to pVpr plus electroporation, 50% of the mice had complete tumor regressions. By day twenty-four post-treatment, 20% (2 out of 10) of pcDNA3.1 plus electroporation treated mice had complete tumor regressions.

Observations were made to determine whether electrically-mediated delivery of pVpr plus electroporation would prolong survival of tumor-bearing mice. We examined the survival of mice after the administration of various treatment regimens with the end point being 100 days after treatment (Figure 15). 50% of the pVpr treated mice that responded to treatment remained tumor-free for one-hundred days post-treatment. Also, there were 2 out of 10 mice treated with 100 µg pcDNA3.1 plus electroporation that were tumor-free for 100 days. The mean survival time for the mice treated with pVpr without electroporation, pcDNA without electroporation, saline with electroporation (electric pulses only), and untreated mice was 10.20, 8.90, 13.40, and 8.40 days, respectively. The mean survival time of mice treated with 100 µg pVpr plus electroporation was 61.80 days. The 100 µg pVpr+ E+ treated mice lived significantly longer ($p<0.05$) than mice treated with 100 µg pVpr without electroporation, 100 µg pcDNA3.1 without electroporation, saline with electroporation, and untreated mice. The mean survival time for 100 µg pcDNA3.1 +E+ treated mice was 38.60 days. In terms of mean survival time, there was no statistical difference between mice treated with 100 µg pVpr+ E+ and 100 µg pcDNA3.1+E+.

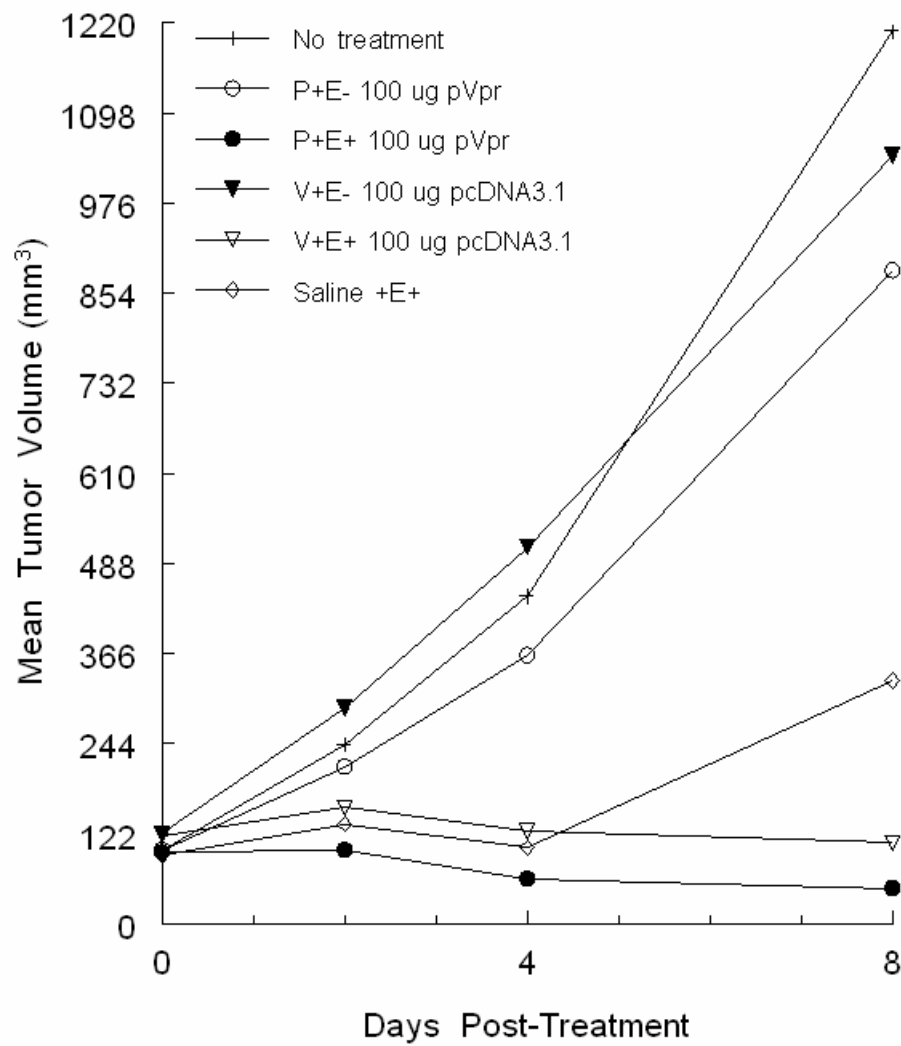


Figure 14. Tumor Volume Reduction After Treatment with 100 μ g pVpr on Days 0, 2, and 4

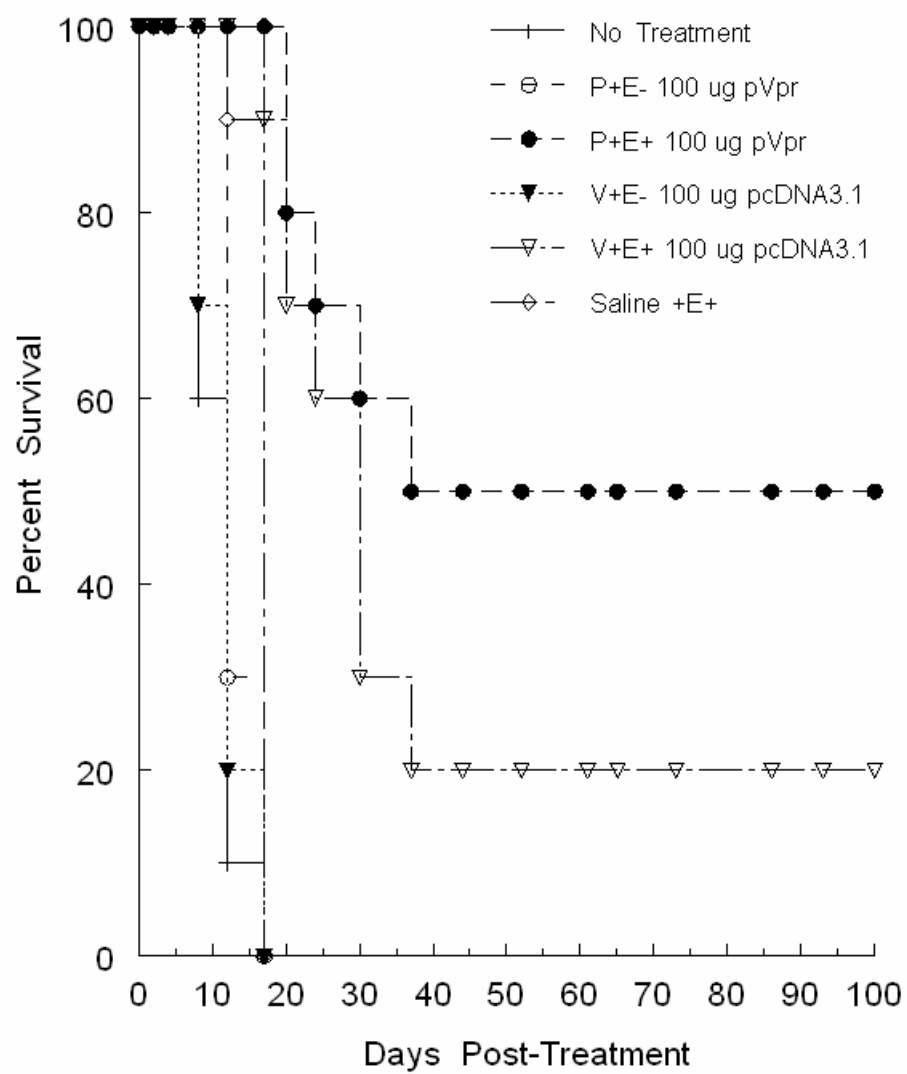


Figure 15. Kaplan-Meier Survival Curve After Treatment on Days 0, 2, and 4

Intratumoral Expression of Vpr *In Vivo* After Multiple Treatments

Previous immunohistochemistry staining demonstrated Vpr expression at day 2 after a single injection of 100 µg pVpr with electroporation but the protein expression diminished by day 4. The current experiment was conducted in order to determine whether multiple treatments of 100 µg pVpr with electroporation would lead to sustained Vpr expression for several days post-treatment. The multiple treatment regimen involved administering 100 µg pVpr plus 1500 V/cm, 100 µs on treatment days 0, 2, and 4. The tumors were sectioned to detect Vpr protein by immunohistochemistry. As early as day 2 post-treatment (1 injection with pVpr plus electroporation on day 0), Vpr protein was detected (Figure 16A). Multiple treatments with 100 µg pVpr plus electroporation led to intense expression of Vpr at day 7 post-treatment (after 3 exposures to 100 µg pVpr plus electroporation) (Figure 16B).

Summary of Specific Aim 1

For the purpose of effectively treating subcutaneous B16 melanoma, certain treatment parameters had to be elucidated during the preliminary phase of experimentation. The parameters that had to be tested were the pVpr dosage, electroporation conditions, Vpr protein expression levels, and the schedule of treatment days. B16 melanoma lesions were treated with dosages ranging from 25 µg to 200 µg of pVpr plus electroporation on day 0 and day 4. Only the mice that received 100 µg pVpr plus electroporation had significant tumor reduction in comparison to the untreated mice. Therefore, the 100 µg pVpr dosage was chosen as the dosage to be further evaluated in subsequent growth inhibition studies in conjunction with electroporation.

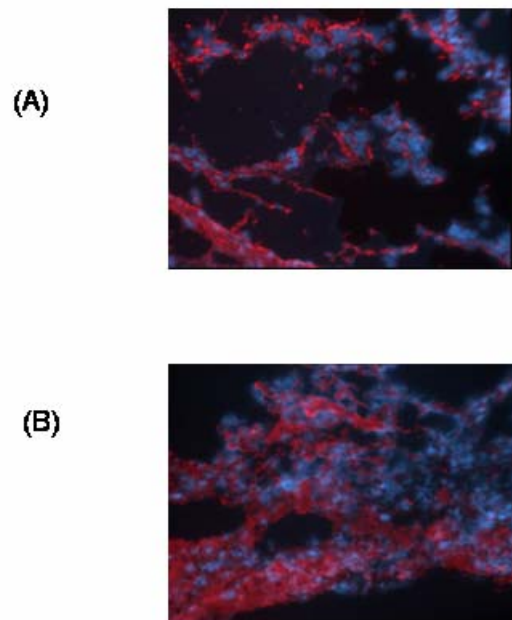


Figure 16. Vpr Expression on Day 2 and Day 7 Post-Treatment. (A) Tumor excised at day 2 post-treatment and stained with PE-conjugated Vpr antibody. (B) Tumor excised at day 7 post-treatment and stained with PE-conjugated Vpr antibody.

The next phase of the study examined four different electroporation parameters to determine which parameters would cause the highest Vpr protein expression after treatment with 100 µg pVpr +E+ on day 0 & day 4. The electroporation parameters that were investigated were 100 V/cm, 150 ms; 200 V/cm, 20 ms; 800 V/cm, 5 ms; and 1500 V/cm, 100 µs. Also, it was found that Vpr protein was expressed two days following treatment and diminished by day 4 following treatment. The electroporation parameters of 1500 V/cm, 100 µs yielded the highest Vpr expression in comparison to the other electroporation parameters delivered with electroporation and pVpr alone. In addition to Vpr expression data, at the early time points (i.e. days 4 and 8), the 100 µg pVpr plus 1500 V/cm, 100 µs treated tumors had the lowest tumor volumes compared to tumors treated with 100 µg pVpr without electroporation and all the other tumors treated with pVpr plus various electroporation parameters.

The concentration of pVpr at 100 µg and the electroporation parameters of 1500 V/cm, 100 µs pulses were sufficient to induce B16 tumor reduction but not durable complete responses. It was anticipated that in order to attain durable complete tumor regression, the schedule of treatment days would have to be revised. The initial treatment regimen involved treatments on days 0 & 4 but new avenues would have to be explored such as daily treatment days or alternating treatment days on the quest to attain complete regressions. Treatments on days 0, 4, 7 and 11 led to mice that lived significantly ($p < 0.05$) longer than untreated mice following treatment with P+E+ 50 µg pVpr or P+E+ 100 µg pVpr. The four day treatment regimen suggested that closer intervals of treatment may be necessary to obtain more reduction in tumor volume.

Closer intervals of treatment were examined with the treatment regimen of consecutive treatment days (i.e. day 0 and 1) or alternating treatment days (i.e. 0 and 2). Initially, the consecutive treatment regimen of pVpr +E+ caused more reduction in tumor volume than the alternating treatment regimen. By the second week after treatment with pVpr +E+, the alternating treatment appeared more effective in reducing tumor volume.

Since the consecutive treatment (i.e. day 0 and day 1) regimen yielded a reduction of tumor growth at the early time points, another experiment was conducted to determine whether daily treatments of pVpr +E+ (i.e. day 0 –day 7) would induce tumor reduction and complete regression. Daily treatments with 100 µg pVpr + E+ once daily or 100 µg pVpr + E+ twice daily led to 80% of mice with durable complete tumor regressions. One mouse had durable complete regression following every other day treatment with 100 µg pVpr + E+. Also, 100 % of the mice had durable complete regressions after treatment with 100 µg pcDNA3.1. Since 80- 100 % of mice treated daily with pVpr + E+ or pcDNA3.1 +E+ had complete regressions, a difference between pVpr and non-coding vector could not be established. Therefore, in order to possibly differentiate between the efficacy of pVpr and pcDNA3.1, a revised treatment regimen was devised. The revised treatment regimen stems from evidence that alternating treatment days of pVpr +E+ led to tumor reduction with the only modification being an additional day incorporated into the regimen (i.e. day 0, 2, and 4).

Once the optimal electroporation parameters were found, several main experiments were conducted to determine the efficacy of tumor reduction and/or regression as a consequence of using the optimal pVpr dosage as well as the optimal electroporation parameters. The set of experiments to demonstrate B16 tumor regression

involved treating mice with 25 μ g or 100 μ g pVpr plus electroporation. The experiments were repeated twice for a total n of 14 for the pVpr +E+ and the pcDNA3.1 +E+ group. By day 14 post-treatment, 42.86% of the mice had complete tumor regressions following treatment with 100 μ g pVpr plus electroporation. Also, by day 14, 14.29% of the mice had complete tumor regressions following treatment with 25 μ g pVpr plus electroporation. Due to residual tumor cells, tumors recurred in the mice that were treated with 100 μ g pVpr plus electroporation which left 7.14% of these mice with complete regression past 100 days post-treatment. Whereas, 14.29% of the mice treated with 25 μ g pVpr+E+ maintained regression past 100 days post-treatment.

Another set of experiments were devised from the concepts developed in the preliminary experiments regarding the alternating treatment regimen (i.e. day 0 and day 2 treatment) for enhanced tumor reduction with tumor regression. The modification in this set of experiments was that treatment was administered on day 0, 2, and 4. The experiments were repeated twice for a total n of 10. Mice were treated with 100 μ g pVpr or 100 μ g pcDNA3.1 with electroporation. After 100 days post-treatment, 50 % of pVpr +E+ treated mice and 20% of pcDNA3.1 +E+ treated mice had complete tumor regressions. Therefore, 100 μ g pVpr with 1500 V/cm, 100 μ s administered on days 0, 2, and 4 yielded the highest percentage of durable complete regressions.

Specific Aim 2

To Elucidate a Mechanism Regarding the Ability of Vpr to Inhibit Tumor Growth In Vivo

Apoptosis is the Mechanism of Tumor Reduction

Previous *in vitro* studies have demonstrated that Vpr protein induces apoptosis in a variety of cancer cell lines (54,83). However, there has not been a definitive *in vivo* study that shows that Vpr protein induces intratumoral apoptosis and has a role in B16 tumor reduction. The tumors used in this portion of the study were treated according to the two protocols for the optimal pVpr +E+ treatment regimen. The first protocol involved treating tumors with the high dose group, 100 µg pVpr +E+ on days 0 and 4. The second protocol involved treating tumors with 100 µg pVpr +E+ on days 0, 2, and 4.

It was important to determine the *in vivo* Vpr expression efficiency and any Vpr induced apoptosis after intratumoral injection of pVpr into established subcutaneous B16 tumors with *in vivo* electroporation. Initially, to address this issue, melanoma tumors were excised 48 hours after a single intratumoral treatment with 100 µg of pVpr delivered with electroporation. After appropriate sectioning and tissue fixation, tumor sections were stained with a rabbit polyclonal anti-Vpr antibody and a TUNEL reaction mixture for measurement of protein expression and apoptosis, respectively (Figure 17). After examination of the sections by fluorescent microscopy under dual filters the areas of fluorescence, indicative of expression of Vpr and apoptosis, were assessed. Figure 17A and 17B show tumor sections from pVpr +E+ treatment and pcDNA3.1 +E+ treatment which were stained with Hoechst reagent, which is a positive control which stains nuclei. Figures 17C and 17D indicate PE conjugated anti-Vpr antibody staining for the pVpr plus

electroporation and pcDNA3.1 control plus electroporation groups, respectively.

Although some background staining is present in the pcDNA3.1 section, staining in the pVpr section is considerably more extensive and intense indicating expression of Vpr. Figures 17E and 17F show overlays of the tumor sections indicating Vpr and Hoechst staining of pVpr plus electroporation and pcDNA3.1 plus electroporation treatment groups, respectively. The panels in Figures 17G and 17H indicate Hoechst staining for consecutive sections from the pVpr and pcDNA3.1 treated tumors and serves as the positive control for nuclei in the TUNEL staining. Finally, Figures 17I and 17J show TUNEL staining (specifically nuclear staining) of sections from tumors treated with pVpr plus electroporation and pcDNA3.1 plus electroporation, respectively. This result demonstrated specific TUNEL staining (fluorescein), indicative of apoptosis, only in the pVpr plus electroporation treatment group. In summary, the results demonstrate specific Vpr expression and apoptosis induced by the Vpr DNA construct when delivered intratumorally, with electroporation, into these aggressive and established subcutaneous B16.F10 tumors. Overall, the data demonstrate the ability of a plasmid expressing Vpr, when delivered electroporatively to B16.F10 tumors, to effectively result in long-term survival of treated mice coupled with complete tumor regression.

Additional studies were conducted to evaluate whether apoptosis is the mechanism by which Vpr inhibits tumor growth and causes tumor regression *in vivo*. Tumors that were exposed to pVpr plus electroporation demonstrated apoptosis as early as two days post-treatment and continued to undergo apoptosis for a number of days following treatment (Figure 18). Hoechst was used as a positive control for nuclei in the PE-conjugated anti-Vpr antibody staining of all the tumor sections. Figure 18 (A-C)

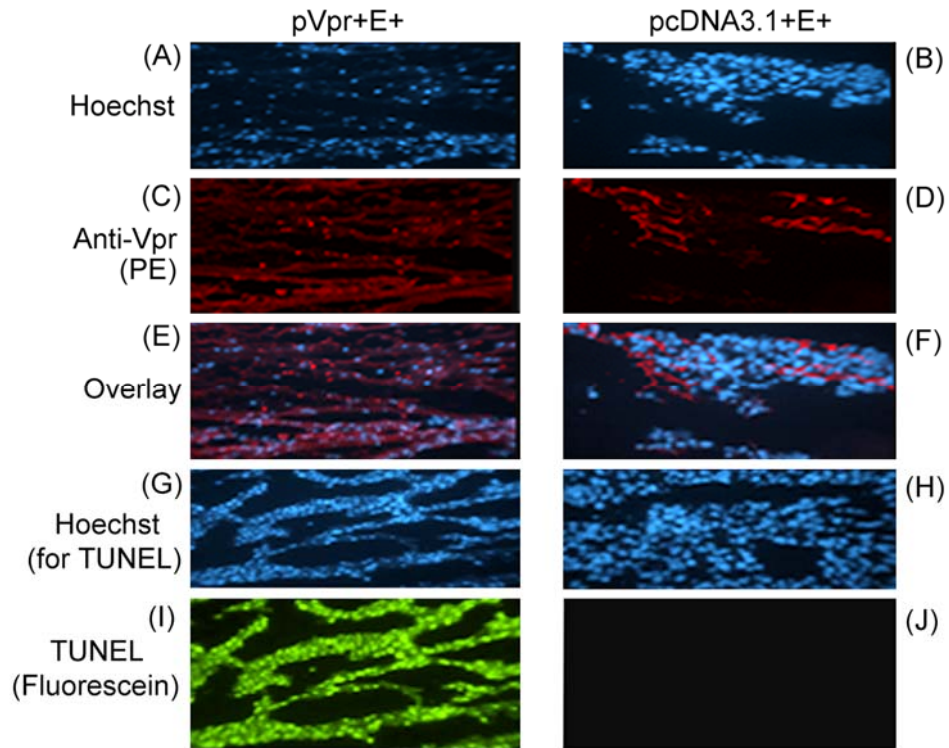


Figure 17. Day 2 *In Vivo* Expression of Vpr and Induction of Apoptosis After pVpr Treatment with Electroporation
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shows tumor sections from pVpr +E+, pcDNA3.1 +E+, and saline +E+ groups, respectively. These results show the overlays of the tumor sections indicating expression of Vpr and Hoechst staining. Figure 18 (D-F) indicates Hoechst staining for sections from pVpr +E+, pcDNA3.1 +E+, and saline +E+ treated tumors, respectively. Hoechst serves as the positive control for nuclei in the TUNEL staining. Figures 18 (G-I) show TUNEL staining of sections from tumors treated with pVpr +E+, pcDNA3.1 +E+, and saline +E+, respectively. This data showed specific TUNEL staining (fluorescein), which signified apoptosis, only in the pVpr +E+ treated tumors. Irrespective of multiple treatments, negligible apoptosis levels, slightly above background levels, were observed in tumors treated with electric pulses only or 100 µg pcDNA3.1 with electroporation. After three treatments, 100 µg pVpr alone induced apoptosis but not as efficiently as electroporation-mediated delivery of 100 µg pVpr (data not shown).

Summary of Specific Aim 2

The purpose of Specific Aim 2 was to elucidate a mechanism regarding the ability of Vpr to inhibit tumor growth and cause regression *in vivo*. Previous *in vitro* studies demonstrated Vpr-induced apoptosis. The current study demonstrated for the first time that Vpr protein caused apoptosis in established B16 tumors. Vpr protein induced apoptosis as early as two days after the intratumoral delivery of pVpr with electroporation. Also, sustained apoptosis corresponded to sustained Vpr expression for up to seven days following intratumoral treatment with 100 µg pVpr +E+ on days 0, 2, and 4. Data suggest that intratumoral apoptosis is the mechanism by which Vpr protein causes B16 tumor regression.

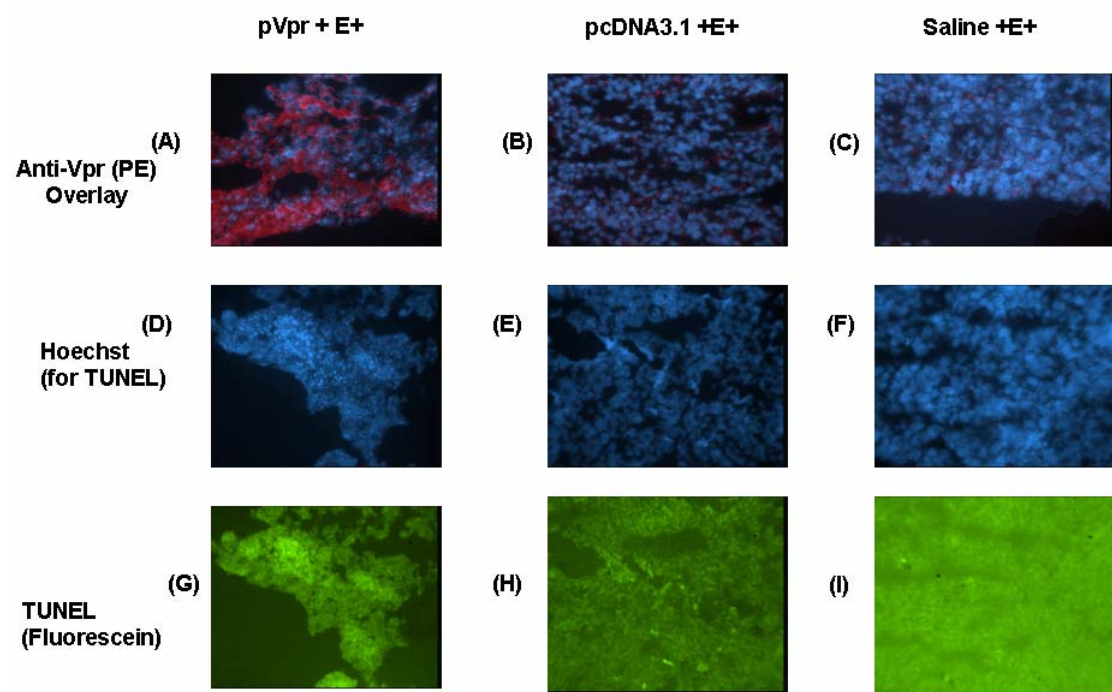


Figure 18. Day 7 Immunohistochemical Staining and Apoptosis Staining of Tumor Treated with Three Treatments of pVpr+E+

Specific Aim 3

To Investigate whether pVpr Induces an Immune Response, which would allow the Treated Mice to be Resistant to Tumor Challenge

Cured Mice Resist B16.F10 Challenge

During the course of conducting research pertaining to electrically-mediated delivery of pVpr to established melanoma, several mice had durable complete regression, cured, of cancer. To examine whether these mice were able to resist future exposure to the parental cancer cells, B16.F10 melanoma cells were injected on the right flank (i.e. the flank opposite to the one that was originally treated). The initial challenge experiments involved injecting 1×10^6 of B16.F10 cells, the original concentration, on the opposite flank of cured mice. The latter challenge experiments involved injecting 5×10^5 B16.F10 cells on the opposite flank of cured mice. Cured mice were challenged after remaining tumor-free for ~ 100 days post-treatment. By using 1×10^6 or 5×10^5 B16.F10 cells in some of the challenge experiments, it was determined that the amount of parental cells does not influence whether cured mice are able to resist tumor challenge. It was customary to inject naïve C57Bl/6 mice with the same batch of B16.F10 cells that were used in the challenge experiments as a control for their viability in the cured mice. All cured mice that resisted tumor challenge were also monitored for continued complete regression at the original treatment site.

Cured Mice Resist Tumor Challenge Following Treatment with pVpr plus Electroporation on Days 0 and 4

Both the low and high dosage pVpr treatment regimen delivered with electroporation yielded cured mice that were able to resist tumor challenge. Following treatment with 25 μg of pVpr +E+ on days 0 and 4 post-treatment, two out of fourteen mice were cured. The two 25 μg pVpr +E+ treated mice were challenged with 1×10^6 B16.F10 cells on the opposite flank (right flank). By day 10 post-challenge, a tumor appeared at the challenge site on one of the 25 μg pVpr +E+ treated mice, whereas the other 25 μg pVpr +E+ treated mouse resisted tumor challenge until the end of the time course at 52 days post-challenge. Following treatment with 100 μg pVpr +E+ on day 0 and day 4, one out of 14 mice were cured. The mouse previously treated with 100 μg pVpr +E+ was challenged with 1×10^6 B16.F10 cells on the opposite flank. The 100 μg pVpr +E+ treated mouse resisted tumor challenge for 41 days post-challenge before a tumor appeared at the challenge site.

An additional experiment with pVpr +E+ administered on days 0 & 4 yielded cured mice which were later challenged with B16.F10 cells. One mouse was cured following treatment with 100 μg pVpr +E+ on days 0 & 4 post-treatment. The 100 μg pVpr +E+ treated mouse resisted tumor challenge for 54 days post-challenge. Two mice were cured following treatment with 100 μg pcDNA3.1+ E+. By day 31 post-treatment, only one of the 100 μg pcDNA3.1+ E+ treated mice resisted tumor challenge until day 54 post-challenge.

Cured Mice Resist Tumor Challenge Following Daily Administration of pVpr plus Electroporation

Mice that were previously cured after receiving various daily treatments of 100 μ g pVpr with or without electroporation were challenged with 1×10^6 B16.F10 cells on the right flank. Following treatment with 100 μ g pVpr +E+ twice daily, four out of five mice were cured. Of the cured mice treated with 100 μ g pVpr +E+ twice daily, two mice resisted challenge for the first eleven days post-challenge. By day 16 post-challenge, all the twice daily pVpr + E+ treated mice succumbed to tumor occurrence at the challenge site. Four out of five mice treated with 100 μ g pVpr +E+ once daily were cured. Only three of the once daily treated mice were challenged with 1×10^6 B16.F10 cells because the other cured mouse in this group was excluded (i.e. euthanized) from the challenge experiment due to a persistent skin irritation. All three mice treated with once daily pVpr +E+ were able to resist tumor challenge until the end of the time course at 39 days post-challenge. One mouse was cured following treatment with 100 μ g pVpr + E+ administered on days 0, 2, 4, & 6. The mouse previously cured with pVpr +E+ on days 0, 2, 4, & 6 resisted tumor challenge for 39 days post-challenge which was the end point for this experiment.

Cured Mice Resist Tumor Challenge Following Treatment with pVpr plus Electroporation on Days 0, 2, and 4

In one of the preliminary experiments, one out of four mice were cured after receiving day 0, 2, & 4 treatments of 100 μ g pcDNA3.1 +E+ and challenged with 5×10^5 B16.F10 cells on the right flank. However, the pcDNA3.1 +E + treated mouse did not

resist tumor challenge. Mice that were previously cured after receiving day 0, 2, & 4 treatments of 100 µg pVpr +E+ were challenged with 5×10^5 B16.F10 cells on the right flank. Day 50 post-challenge is the end point for the experiment. Following treatment with 100 µg of pVpr +E + on days 0, 2, and 4 post-treatment, five out of ten mice were cured. At day 11 post-challenge, four mice resisted tumor challenge. On days 39 to 50 post-challenge, three mice resisted tumor challenge. Following treatment with 100 µg of pcDNA3.1 +E + on days 0, 2, and 4 post-treatment, two mice were cured. At the day 11 post-challenge time point, one of pcDNA3.1+ E+ cured mice already had a tumor that had developed for over a week. The other pcDNA3.1+ E+ cured mouse resisted tumor challenge for 50 days post-challenge.

Summary of Specific Aim 3

The purpose of Specific Aim 3 was to determine whether pVpr induces an immune response, which would allow the treated mice to be resistant to tumor challenge. Cured mice were challenged with parental B16.F10 cells after remaining tumor-free for ~100 days post-treatment. After mice were treated with 25 µg pVpr +E+ or 100 µg pVpr +E+ on days 0 and 4, 2 out of 14 mice or 1 out of 14 mice resisted tumor challenge, respectively, for at least 41 days. Three out of five mice and one out of five mice resisted tumor challenge following pVpr +E+ treatment delivered once daily and every other day, respectively. Following treatment with pVpr +E+ on days 0, 2, and 4, three out of 10 mice resisted tumor challenge. Following treatment with pcDNA3.1+E+ on days 0, 2, and 4, one out of 10 mice resisted tumor challenge.

It is important to point out that naïve mice usually develop tumors within one week of tumor challenge and succumb to tumor burden between days 15 and 20. Also, all

of the challenged mice survived at least 39 days after challenge. In some instances mice survived 52 days after challenge. Therefore, the cured mice that were able to resist B16 challenge lived tumor-free for over a month after exposure to the parental B16.F10 cells. This finding suggests that the pVpr plus electroporation treatment most likely elicited an immune response which is involved in protecting challenged mice from tumor development at a time point where there would be significant tumor burden in naïve animals.

Discussion

The B16.F10 murine melanoma model was selected for this study because this tumor cell line has been extensively characterized and is a very good model for many human malignancies due to its highly invasive and metastatic nature. In particular, B16.F10 melanoma is a good model for human melanoma because it is poorly immunogenic due to the lack of MHC I expression and its tumor associated antigens are recognized as self antigens (29,30). It is difficult to attain complete tumor regressions, coupled with long-term survival, through therapeutic interventions in established subcutaneous B16 tumors. The main reason that untreated B16.F10 tumors grow so aggressively is that mice are incapable of mounting a proper immune response against this poorly immunogenic tumor.

B16.F10 melanoma is characteristically difficult to treat or cure once visible tumors have developed. Most researchers are only able to obtain B16 tumor growth delay for a period of time before the tumor burden necessitates euthanization of the animal. Typically in this model any therapeutic intervention resulting in only tumor growth delay is considered to be significant. Usually, untreated B16 tumor-bearing mice die by day 21-25 after inoculation of cells. In addition, it is significant that in the current study the treatment regimen was delivered to established tumors rather than administered either concomitantly with initial tumor cells or before tumors had visibly formed. The qualities of B16 melanoma contribute to its attractiveness as a model for human tumors.

Many investigations have determined that the HIV-1 accessory protein Vpr induces apoptosis in a large variety of tumor cells *in vitro* (34,35,102). While many studies examined the anti-cancer activity of Vpr *in vitro* concurrent with a mechanism of cell killing, there was little research to address the anti-cancer effects of Vpr *in vivo*. There were a few exceptions in which anti-cancer activity of Vpr was examined in mouse models.

Eventually, the anti-cancer activity of Vpr was examined in a solid tumor model. Lentiviral delivery of Vpr to AT-84 oral cancer in a mouse model demonstrated the capability of Vpr to cause tumor regression (103). In the AT-84 study, low infectivity rates of lentiviral vector carrying Vpr led to 10% of transfected cells that were apoptotic *in vitro*. However, in the AT-84 study, tumors that were exposed to Vpr were shown to have regions of dead cells but there was no distinction as to whether the dead cells arose because of necrosis or apoptosis.

There are some safety issues concerning the use of lentiviral vectors in clinical settings. One negative aspect of using lentiviral vectors is that there could be potential adverse reactions if multiple lentiviral treatments were administered in an effort to optimize the treatment regimen. Another negative aspect is that lentiviral delivery could cause insertional mutagenesis in the host genome (104). The AT-84 study showed the feasibility of using the Vpr gene product to slow tumor growth and cause tumor regression in a mouse model. But the study did not provide a gene delivery system that could be safely translated into future clinical utility. Even though *vpr* could be inserted into another viral vector such as an adenoviral vector, in the case of multiple treatment administrations, it would be difficult to circumvent immune reactions due to multiple

exposures to the viral vector. An effective non-viral delivery method such as electroporation would offer a way avoid immune responses to viral vectors.

Specific attention has been focused on the impact that Vpr has on B16.F10 cells. There was a study that investigated the role of Vpr on B16.F10 tumor cell proliferation in an animal model but the study was performed by transfecting the B16 cells with Vpr plasmid prior to injecting the cells into mice. The previous investigation reported that B16.F10 melanoma cells, when transfected with a plasmid expressing Vpr, were significantly less efficient in inducing tumor colonies in the lungs of C57BL/6 mice following intravenous injection (105). The study reported here is the first to investigate the impact of Vpr on established subcutaneous B16.F10 melanoma.

The hypothesis of this study: *Intratumoral delivery of a Vpr expression plasmid administered with in vivo electroporation will lead to growth inhibition and regression of established B16.F10 melanoma in syngeneic C57BL/6 mice.*

The successful use of electroporation to transfer plasmid to cells *in vitro* and the fact that electric fields can be safely and efficiently administered *in vivo* to deliver small molecules offered a basis for the delivery of plasmid via *in vivo* electroporation (106,107). Several studies have demonstrated that electrically mediated delivery of plasmids which encode and express therapeutic molecules can be directed efficiently to tumors. Examples involving experimental melanoma treatment indicated that delivery of plasmids encoding GM-CSF and IL-2, IL-12, tumor antigens and IFN- α elicit an anti-tumor effect (95,98-101,108,109).

In terms of electrogenetherapy, complete tumor regressions of established B16 tumors have been attained mostly with the use of plasmids encoding for cytokines.

Furthermore, the most efficient transfection method to induce complete B16 regressions has been *in vivo* electroporation. Therefore, *in vivo* electroporation was utilized as a delivery method in order to maximize the potential for a therapeutic effect of Vpr when administered as an expression plasmid.

In the study reported here, preliminary data demonstrated that after the electrically-mediated delivery of pVpr, intratumoral Vpr expression lasted for two days after a single administration of treatment. It was later discovered that intratumoral Vpr expression caused intratumoral apoptosis at two days post-treatment. Therefore, various treatment regimens were explored in order to obtain a sustained level of Vpr expression while at the same time improving the ability of Vpr to reduce B16 tumor volume and/or induce complete regression. For these reasons treatment regimens such as daily treatments (i.e. day 0- day 7) and alternating treatments (i.e. day 0, 2, and 4) were examined. With the daily treatment regimen there was no way to differentiate between Vpr induced or non-coding vector induced complete tumor regressions considering that 80 – 100% of treated mice had complete regressions within the same timeframe. Therefore, the alternating treatment regimen (i.e. day 0, 2, and 4) resulted in 50% of the mice having complete regressions after pVpr +E+ treatment and 20% of the mice having complete regressions after pcDNA +E+ treatment. At least with alternating treatments, a distinction could be made between the time points at which complete regression occurred for the pVpr +E+ treated mice and the pcDNA + E+ treated mice. Mice treated with pVpr +E+ on days 0, 2, & 4 had complete tumor regressions by week two after treatment. Whereas, mice treated with pcDNA +E+ on days 0, 2, & 4 had complete tumor regressions by 3- 4 weeks after treatment. Thereby, a distinct mechanism of tumor

regression could be proposed for the pVpr treatment and the non-coding treatment administered on alternating treatment days. Upon further investigation it was found that the tumors treated with multiple days of pVpr+ E+ underwent apoptosis, whereas the tumors treated with multiple days of pcDNA3.1 +E+ did not experience apoptosis.

In this study, Vpr has shown great potential as an anti-tumor agent in terms of attenuation of B16 tumor growth and complete tumor regressions. During the first twelve days following treatment, mice that received day 0, 2, & 4 treatments of 100 µg pVpr with electroporation had substantial reductions in tumor volume in comparison to the other treatment groups. By day 12 post-treatment, the tumor reductions translated into complete tumor regressions in 50% of the pVpr plus electroporation treated mice. The enhanced expression of Vpr correlates with greater apoptosis within the tumor which would allow for greater reduction in tumor volume.

The Vpr protein is a fast-acting protein which is produced within 48 hours following a single electrically-mediated pVpr injection. Tumors that were initially treated with pVpr plus electroporation not only demonstrated apoptosis as early as 48 hours post-treatment but continued to undergo apoptosis for a number of days following three treatments. After multiple exposures to pVpr plus electroporation, Vpr –induced apoptosis could possibly enhance the ability of the immune cells to recognize the B16 tumor antigens derived from apoptotic B16 tumors. It is a possibility that after treatment with pVpr+ E+, there could be improved availability of B16 antigens which might increase the likelihood that this ordinarily poorly immunogenic tumor would be recognized by antigen presenting cells which in turn would be engaged by cytotoxic T cells to destroy the tumor (110,111).

It is commendable that Vpr had the capability to induce tumor regressions through an apoptotic-mediated mechanism in the B16.F10 melanoma model which is known for its highly aggressive nature and tendency to recur even after complete regressions occur. Therefore, due to its apoptosis-inducing properties, it is anticipated that the pVpr +E+ treatment would be effective in eradicating less aggressive tumors which could result in higher percentages of complete regressions.

It is important to note that gene delivery via electroporation enhanced the Vpr gene expression as evidenced by the tumors treated with pVpr +E+ producing a sufficiently high level of Vpr protein to induce apoptosis. At low levels of expression, Vpr protein will not cause apoptosis, thereby Vpr protein will exhibit an anti-apoptotic effect (79,80). There was a possible anti-apoptotic effect exhibited when tumors treated with pVpr without electroporation expressed Vpr protein but not at sufficiently high levels to cause apoptosis (data not shown). Even when pVpr was administered without electroporation on three treatment days, pVpr alone did not cause substantial apoptosis. Whereas, after a single treatment with pVpr +E+, apoptosis was induced. The current study definitively showed that the delivery of pVpr via electroporation allowed for a high level of Vpr protein expression. The high level of Vpr protein expression led to B16 tumor regression which was mediated through apoptosis.

The pVpr + E+ treatment regimen not only eradicates the primary B16 melanoma but can offer protection against future B16 melanoma development. Once mice had complete tumor regression for approximately 100 days after treatment they were considered cured of B16 melanoma. The cured mice were challenged with B16.F10 cells to determine whether they developed an immune response against the tumor cells. After

mice were treated with 25 µg pVpr +E+ or 100 µg pVpr +E+ on days 0 and 4, 2 out of 14 mice or 1 out of 14 mice were cured and resisted tumor challenge for at least 41 days. Following the daily treatment regimen there were some mice that were able to resist tumor challenge in the groups that were previously cured with once daily 100 µg pVpr +E+ and every other day with 100 µg pVpr +E+. Following treatment with pVpr +E+ on days 0, 2, and 4, three out of 10 mice were cured and resisted tumor challenge. Following treatment with pcDNA3.1+E+, two out of 10 mice were cured but only one of these mice resisted tumor challenge. Previous studies have indicated that under the right conditions a high percentage of mice experience long-term protection against parental tumor following treatment with either an electrically-mediated non-coding vector or CpG-ODN (112,113). However, in the current study, long-term protection against B16 melanoma occurred more often in mice cured with one of the pVpr+ E+ treatment regimens as opposed to the pcDNA3.1 +E+ treatment regimen.

In some instances, there were durable complete regressions observed after treatment with either pVpr or pcDNA3.1 plus electroporation. The data from the daily pVpr or pcDNA3.1 plus electroporation treated tumors indicate, even though both treatment regimens resulted in complete tumor regression, the Vpr exposed tumors responded by 11-12 days post-treatment in the once daily group, the group that received pVpr+E+ on days 0, 2, 4, and 6, and group that received pVpr+E+ on day 0, 2, & 4. Whereas, the pcDNA+E+ treated tumors had complete tumor regression by day 18 -24 days post-treatment. In addition, 50% of the pVpr+ E+ treated mice had regressions from day 12 post-treatment until day 100 post-treatment with pVpr +E+ on days 0, 2, and 4. The non-coding vector effect (i.e. pcDNA3.1+ E+) took approximately three weeks to

cause regression in a minimal number of mice with the remainder of these mice dying by ~ day 30 post-treatment. Even though 2 out of 10 pcDNA+E+ treated mice remained tumor-free for 100 days, a greater percentage of mice exposed to pVpr +E+ remained tumor-free for 100 days. The difference in timing of regression and the fact that pcDNA+E+ treatment induces only minimal intratumoral apoptosis, suggests that Vpr-induced tumor regression and pcDNA-induced tumor regression occurs based on two different mechanisms. A possible mechanism for pcDNA induced regression is immunostimulatory CpG motifs in the vector backbone.

By using electrically-mediated delivery of plasmid encoding for Vpr or a non-coding plasmid, this research demonstrates two different pathways to induce complete tumor regression of a poorly immunogenic, aggressive tumor. The first pathway involves inducing apoptosis via intratumoral Vpr protein expression. The second pathway involves evoking an immune response against tumor antigens. Interestingly, both pathways result in lifelong immunity against B16 melanoma in this mouse model.

Previous research involving CpG-ODNs (oligodeoxynucleotides which include CpG motifs) demonstrated that basic peritumoral injections of CpG-ODNs alone had the capability of eradicating established tumors and inducing long-term immunity against the parental tumor (112,114). It is feasible to speculate that electroporation more efficiently delivers the non-coding plasmid containing CpG motifs directly into the tumor, thereby possibly allowing eradication of the tumor in a similar manner as the CpG-ODNs. An earlier electroporation study indicated that delivering non-coding vector with electroporation caused complete tumor regression and long-term immunity in an established B16 melanoma (113). An additional study suggests that a possible source of

tumor regression in non-coding vector treated mice may be immune cell infiltration due to the CpG motif in the vector (115). Also, perhaps, the more efficient delivery of the non-coding plasmid induced complete tumor regression in the current study after 2-7 injections of plasmid with electroporation, whereas, the delivery of CpG-ODNs required 15 basic injections to obtain complete tumor regression in a solid tumor (112). Even though established neuroblastoma is a difficult tumor to treat, the use of CpG-ODNs offered a way to cure tumor-bearing mice from this solid tumor (112,116). Therefore, the CpG motifs could contribute to the cure of the B16 solid tumor in the current study.

The mechanism by which CpG-ODNs induce tumor regression involves NK cells, dendritic cells, macrophages, and T cells. There have been recent investigations concerning the use of CpG-ODNs in the treatment of melanoma in a pre-clinical melanoma model (117,118). Delivery of non-coding plasmid with electroporation is less labor intensive than CpG-ODNs synthesis and may have future utility in treatment of cancer via non-specific as well as tumor-specific immunity.

This study initially demonstrated the feasibility of using a plasmid encoding for Vpr delivered by *in vivo* electroporation to successfully treat established B16 tumors, which resulted in growth inhibition and ultimately regression. This is the first comprehensive study demonstrating the ability of Vpr, when delivered as a plasmid via *in vivo* electroporation, to induce complete tumor regressions in a solid tumor. Even though a lentiviral vector carrying Vpr demonstrated tumor regression in AT-84 tumors, there are safety concerns regarding the use of lentiviral vectors in the clinical setting. The current study is the first one to deliver pVpr by *in vivo* electroporation which allows for multiple treatments without potential immune reactions. As the study continued, it

showed that the tumor inhibition and regression corresponded to the intratumoral Vpr-induced apoptosis. Previous research had only shown Vpr protein expression and its resultant apoptosis *in vitro*. The current study offers one of the first evidences which demonstrate *in vivo* apoptosis as a result of Vpr protein expression. Also, this study was the first to demonstrate an optimized treatment regimen by incorporating closer intervals between treatments in order to attain higher percentages of B16 tumor regressions. In the future, after additional pre-clinical studies, electrically-mediated pVpr may be efficacious against human cancer.

The ultimate therapeutic endpoint was complete tumor regression and long-term survival rather than simply attenuation of tumor growth which has been the benchmark of most other studies. Therefore, the percentages of the complete tumor regression coupled with long-term survival attained in this study with pVpr plus electroporation treatment are biologically significant and warrant further mechanistic studies as well as the assessment of methods to maximize the anti-tumor effect through the use of different plasmid doses and Vpr treatment regimens. In addition, once the anti-tumor effect can be further maximized for the pVpr+ E+ treated mice, potential immune mechanisms, such as potential presentation of specific melanoma tumor antigens released by the apoptotic process, could be further investigated. Overall, the data presented underscore the therapeutic anti-tumor activity of both Vpr and the electroporation delivery method and suggests a potential clinical utility for this treatment modality against melanomas as well as possibly other tumor types. In sum, the data suggest that gene delivery using Vpr provides a way to harness the cell killing potential of Vpr in HIV infected hosts and utilize it against malignant tumors such as melanomas.

Future Directions

Completion of this study established an understanding of the anti-tumor effects of intratumoral delivery of a Vpr expression plasmid mediated through *in vivo* electroporation. This study also demonstrated apoptosis as a mechanism of action for Vpr protein induced tumor regression in the B16 tumor model. This study serves as a foundation for future studies to investigate the further implications of the capability of Vpr protein to cause growth inhibition and regression in solid tumors.

Further experiments should be proposed to extend our understanding of how Vpr induces tumor regression. For example, experiments should be conducted to determine if and to what extent intratumoral immune cells such as T cells, NK cells, dendritic cells, and macrophages contribute to tumor regression in B16 tumors that have been treated with pVpr +E+. Another approach to determine the importance of T-cell mediated immunity in Vpr induced tumor reduction would be to use a nude mouse model.

It would be interesting to further evaluate whether there are anti-proliferative effects after the delivery of pVpr plus electroporation in other tumor models especially human melanoma. In this case, human malignant melanoma cells such as A375 cells would be injected into nude mice in order to establish a tumor. Subsequently, the A375 tumor would be treated with pVpr+E+. The nude mouse model would provide a way to

determine the capability of Vpr protein to have anti-tumor activity in the context of the innate immune system.

It would be interesting to examine other apoptosis-inducing HIV-1 accessory proteins in the context of the B16 established tumor. Since B16.F10 is highly aggressive, it would be a good tumor model to test any anti-tumor activity of other HIV-1 accessory proteins. Experiments could be conducted to determine whether electrically-mediated delivery of other accessory HIV-1 genes would have the capability to inhibit B16 growth or cause B16 tumor regression. Thus far, Nef has been shown to cause growth inhibition in a tumor model (119). It may even be efficacious to combine Vpr with one of the other HIV-1 accessory proteins to test for synergistic anti-tumor capabilities.

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